REMARKS/ARGUMENTS

Status of the Claims

Claims 5, 7-14 and 16-18 were withdrawn from consideration by the Examiner in the Office Action. Claims 1-4, 6 and 15 are pending and under consideration.

Claims 19-23 were newly added. The new claims find support throughout the specification, for example, in paragraphs 75-84, 112-125, or Example 2. The new claims do not add new matter.

Amendment of Claims

Claim 1 was amended to include specific steps that characterize the method of utilizing an integrin α10 chain or an integrin α10 chain and an integrin α11 chain as a marker for mammalian mesenchymal stem cells (MSCs). This amendment finds support throughout the specification, for example, in the abstract, paragraphs 63-84, and the originally filed claims.

Claim 3 was amended to include a "contacting step" that involves a molecule which specifically binds to integrin α10 chain or integrin α10 chain and integrin α11 chain. This amendment finds support throughout the specification, for example, in paragraphs 75-84 and 125.

Claims 4 and 6 were amended to adapt the claims to amended claim 3 on which they depend.

Claim 15 was amended to include the step of detecting the specified marker to identify the mammalian mesenchymal stem cell. This amendment finds support throughout the specification, for example, in paragraphs 30-37.

None of the amendments added new matter.

Rejection under 35 U.S.C. § 112, second paragraph

Claims 1-4, 6 and 15 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. *Office Action, page 3.* More specifically, claims 1 and 15 were held to be indefinite for failing to recite active, positive steps that characterize the claimed methods. Applicants respectfully traverse. In response, however, in order to expedite prosecution, Applicant has amended claims 1 and 15 to recite specific steps that characterize the claimed method of using a marker for mammalian MSCs.

Furthermore, claim 3 was held to be indefinite for failing to include at a minimum a contacting step, a detection step, and a correlation step. In response, Applicant has amended claim 3 by adding a "contacting step". The amended claim now includes a contacting step, a detection step, and a correlation step.

In light of the above, Applicant respectfully requests that the rejections under 35 U.S.C. § 112, second paragraph, be withdrawn.

Rejection under 35 U.S.C. § 112, first paragraph

Claims 1-4, 6 and 15 were rejected under 35 U.S.C. § 112, first paragraph, as lacking enablement.

The Office questioned that Example 3 of the specification shows expression of integrin α10 in MSCs. *Office Action, page 4.* Applicant respectfully disagrees for the following reasons.

Example 3 Demonstrates MSCs Express Integrin α10

Isolated bone marrow stromal cells cultured on plastic yield a heterogeneous cell

population containing MSCs as well as more differentiated derivative cells and monocytes/macrophages. The ratio of the various cell types depends on the length and the conditions of *in vitro* culture. For example, while the multipotency and the associated *in vivo* bone forming capacity of *in vitro* cultured bone marrow stromal cells is initially high, these properties are reduced dramatically upon *in vitro* expansion beyond reaching confluence for the first time. *See, for example, Bianchi et al., Exp. Cell Res. 287:98-105 (2003).* Multipotency is lost with increasing passage number. The fraction of MSCs in the *in vitro* cultures, and therefore multipotency and *in vivo* bone forming capacity, can be maintained at a high level by the addition of FGF-2 to the culture medium. Moreover, MSCs can be enriched by addition of FGF-2 to *in vitro* bone marrow stromal cell cultures. Enrichment occurs because FGF-2 is a potent mitogen for MSCs (while maintaining their multipotency) and may also induce apoptosis in differentiated cells.

These points are illustrated in the study by Bianchi et al. who demonstrated that FGF-2 supplementation of primary bone marrow stromal cell cultures *in vitro* selects for the survival of multipotent MSCs, which are capable of differentiating into various different cell types, including the three major derivatives, i.e. osteoblasts, chondrocytes, and adipocytes. *Id.* As a result, FGF-2 led to an enrichment of MSCs in *in vitro* bone marrow stromal cell cultures. Another study also showed that FGF-2 increases the growth rate and life span of MSCs, and that incubation with FGF-2 maintains the multipotency of MSCs throughout many cell divisions. *Tsutsumi et al., Biochem. Biophys. Res. Commun. 288:413-9 (2001).* FGF-2 has similar effects on proliferation

and self-renewal of MSCs isolated from other sources, such as adipose tissue. *See, for example, Zaragosi et al., Stem Cells 24:2412-9 (2006).*

In Example 3 of the instant application, MSCs were isolated using a method essentially as described in paragraphs 90 to 97 of the specification. This method involves density centrifugation of primary bone marrow cells and subsequent culture of nucleated cells on plastic dishes. Only the stromal cells (MSCs and derivative cells) and not the hematopoietic cells (except monocytes/macrophages) of the bone marrow have the capacity to adhere to the plastic. The MSC cultures were then grown for 12 days with repeated changes of growth medium and subculture, consistent with the disclosed method (see paragraphs 96 to 97). At this point in the procedure, MSCs were successfully isolated according to the disclosed method.

The goal of the experiment in Example 3 was to show that the colony forming cells derived from the bone marrow represent MSCs and that these MSCs express integrin α10 (see paragraphs 228-229). For this purpose, the cells were cultured for an additional two weeks, whereby one fraction remained without FGF-2 while the other fraction was cultured in the presence of FGF-2. The rational of this experimental setup was that FGF-2 would maintain and enrich the MSCs in the latter fraction, while some of the MSCs in the former fraction might begin to differentiate and loose their multipotency due to the prolonged culture and the absence of FGF-2 (see above). Subsequent to the additional two week culture the cells were analyzed for known markers, morphology and expression of integrin α10. Only the results for the cells cultured in the presence of FGF-2 are shown in Figure 4. As expected, the enriched MSCs tested negative for non-MSC markers and showed colony morphology typical of MSCs (see paragraphs 238-

239), confirming that the cells in the FGF-2 treated culture were MSCs. Furthermore, 96% of the cells in the MSC enriched fraction expressed integrin α10 (see Figure 4). This result therefore convincingly demonstrated that integrin α10 is a marker for MSCs.

Figure 4 Shows 96% of the MSCs Express Integrin α10

Applicant respectfully disagrees with the Office's interpretation of Figure 4, including for the following reasons. The Office stated that "cells treated with EGF-2 formed colonies typical of MSCs expressed the integrin alpha10 (see figure 4b), while the control did not." *Office Action, page 4.* However, the specification clearly states that "96% of the cells treated with FGF-2 expressed the integrin alpha10" and that "the cells formed colonies typical of MSCs" (see paragraphs 237 and 239). In addition, the cells tested negative for non-MSC markers CD34 and CD45 (see paragraph 238). "Cells" or "the cells" as used in these paragraphs refers to the FGF-2 treated MSCs used in the experiment shown in Figure 4. The cells used in Figure 4a and 4b are the same.

The control shown in Figure 4a is the standard isotype control that is normally used in FACS analyses. In such an isotype control, the specific primary antibody (here the integrin α10 monoclonal antibody) is omitted during FACS analysis. Only the non-specific secondary antibody is added to such a control (see paragraph 238). Such a control uses the same cells as the sample to be analyzed with the specific antibody and it ensures that the positive signal observed in the sample (shown here in Figure 4b) is due to the activity of the specific antibody and not to any non-specific events.

Hence, Applicant sees no scientific basis for the Office's contention that the cells in the control (Figure 4a) are different from the cells in the analyzed sample (Figure 4b) with respect to integrin α10 expression.

The Office also concluded that "it is clear from Applicant example that the starting material (i.e., mesenchymal stem cells) do not express alpha 10 integrin on the cell surface (see Fig.4)." *Office Action, page 4.* However, as explained above, Figure 4b (in conjunction with the isotype control in Figure 4a) clearly demonstrated that 96% of the FGF-2 treated MSCs express integrin α10 on their cell surface. The 4% of cells that did not express integrin α10 on their cell surface may reflect a certain level of heterogeneity that is found in any stem cell preparation due to the inherent plasticity of stem cells. In addition, integrin α10 expression on the surface of MSCs is also shown in Example 1 and Figure 2.

The Office Mischaracterizes Murdoch et al.

The Office further referred to the study by Murdoch et al. (European Cells and Materials 6:17 (2003)), apparently to support the contention that the FGF-2 treated MSCs represent a special subpopulation of MSCs that is characterized by a robust chondrogenic response. *Office Action, page 4.* However, Murdoch et al. provide no evidence for and do not suggest that FGF-2 treatment of bone marrow mesenchymal cells results in a special subpopulation of MSCs. Rather, the results reported by Murdoch et al. are entirely consistent with the general enrichment of MSCs in a heterogeneous bone marrow stromal cell population in response to treatment with FGF-2 (see above). Murdoch et al. in fact indicate this in the second to last sentence of their "Discussion & Conclusions" section. The capability to undergo chondrogenic differentiation under conducive conditions is a normal property of MSCs (see Figure 1 of the instant application). After expanding bone marrow mesenchymal cells with or without FGF-2 Murdoch et al. subjected the cells to a chondrogenic differentiation assay

that included pellet culture (instead of monolayer culture) and a chondrogenic medium designed to drive the differentiation of MSCs towards a chondrogenic cell fate. Such a chondrogenic differentiation assay is described, for example, in Bosnakovski et al., Exp. Hematol. 32:502-9 (2004). Hence, the measurement of chondrogenic differentiation by Murdoch et al. is nothing more than the assessment of chondrogenic differentiation potential -- a hallmark of MSCs -- in a heterogenous cell population. The results do not suggest the existence of a special subpopulation of MSCs.

The Office further stated that "it is not clear that osteogenic, myogenic, marrow stroma, tendogenic/ligamentogenic cells of the hMSC express alpha10 integrin," apparently to support the contention that MSCs do not express integrin α10 and therefore the claimed method of utilizing integrin α10 as a marker for MSCs can not work. *Office Action, page 4.* However, Applicant notes that osteogenic, myogenic, marrow stroma, tendogenic/ligamentogenic cells are not MSCs since they are differentiated cells. They are differentiated derivatives of MSCs. Hence, these cells do not have to express integrin α10 for the claimed method to be successful.

The Office also questioned whether surface-expressed integrin α10 can be used as a marker for MSCs and whether the detection of surface-expressed integrin α10 can be used to identify human MSCs, since integrin α10 is expressed in cells other than MSCs. *Office Action, page 4.* However, it is demonstrated in Example 1 -- as acknowledged by the Examiner -- that integrin α10 is expressed on the surface of human MSCs. It is further known that integrin α10 has a non-ubiquitous, restrictive expression pattern in mammalian tissues. *See, for example, Camper et al., Cell Tissue Res. 306:107-16 (2001).* Integrin α10 protein expression has not been detectable in

many of the tissues tested, including testis, liver, spleen or brain. *Id. at 113-114.* Hence, surface-expressed integrin α10 can be used as a marker to distinguish MSCs from all cells that do not express this integrin chain. Applicant's results also revealed that surface-expression of integrin α10 is detectable only on very few cell types besides MSCs, i.e. the expression pattern is highly restricted, and that its expression correlates with multipotency of MSCs. Integrin α10 is the only marker known to Applicant that displays such a correlation, rendering this marker highly valuable for the detection and isolation of multipotent MSCs.

Lehnert Did Not Investigate Expression of the Integrin α10 Protein

The rejection under 35 U.S.C. § 112, first paragraph, also relies on the teaching by Lehnert et al. that integrin α10 is widely expressed in a panel of 24 tissue types. *Office Action, page 4.* Applicant notes that Lehnert et al. determined only expression of integrin α10 mRNA but did not investigate expression of integrin α10 protein. It is widely accepted, however, that mRNA expression does not necessarily correlate with protein expression, and this has been found to be particularly true for integrin α10 expression. *See, for example, Camper et al., Cell Tissue Res. 306:107-16 (2001).* Camper et al. found that integrin α10 mRNA expression was readily detectable in testis, liver, spleen and brain, but integrin α10 protein expression was not detectable in these same tissues. *Id. at 113-114.*

Moreover, Lehnert et al. observed two major transcripts of integrin α10, one of which, a 1.8 kb transcript, is too short to encode a full length integrin α10 protein (see first paragraph of Discussion, page 243). While it may encode a "truncated variant with an altered function", as speculated by Lehnert et al., it has not been demonstrated that

this truncated transcript expresses any functional integrin α10 fragment. Furthermore, Figure 5 of Lehnert et al. (referred to by the Examiner) shows the PCR amplification of an about 500 bases long fragment from the integrin α10 transcripts in various tissues. Since this amplified fragment may only reflect the presence of the truncated transcript described above, it is unclear how this fragment relates to integrin α10 protein expression.

Applicant also notes that analysis of mRNA levels by PCR amplification from total RNA isolated from tissues does not distinguish between the different cell types within the tested tissues and it is therefore unclear whether the observed expression of integrin α10 mRNA occurs only in mesenchymal cells within these tissues or in any other cell types.

The Office further cited WO99/51639 as teaching that integrin α10 is expressed in aorta (normal nonatherosclerotic artery). *Office Action, page 4*. Applicant notes that this study also analyzed exclusively integrin α10 mRNA levels but not integrin α10 protein levels. Hence, for the reasons outlined above the described results may not correlate to integrin α10 protein expression in aorta.

Overall, the results in Lehnert et al. (Figure 5) and WO 99/51639 (Figure 12) revealed that integrin α10 expression is not detectable or barely detectable in numerous tissues, even at the mRNA level and even when tested with nested PCR, one of the most sensitive and specific methods of PCR amplification, as used by Lehnert et al. Hence, both Lehnert et al. and WO 99/51639 showed a non-ubiquitous expression pattern of integrin α10, even at the mRNA level, whereby the highest levels of integrin α10 mRNA were observed in tissues of mostly mesenchymal origin, such as heart,

muscle and aorta. As outlined above, integrin α10 protein expression is much more restricted yet.

In conclusion, Applicant submits that the specification, in conjunction with the art at the time of filing, clearly shows that integrin α10 protein is expressed in MSCs but not in many other cell types, and that integrin α10 is therefore suitable as a marker for the detection or isolation of MSCs. No undue experimentation would be required to practice the invention. In light of this, Applicant respectfully traverses the rejections under 35 U.S.C. § 112, first paragraph, and requests that these rejections be withdrawn.

Rejection under 35 U.S.C. § 102(b)

Claims 1-4, 6 and 15 were rejected under 35 U.S.C. § 102(b) as being anticipated by WO99/51639. Applicant respectfully traverses.

The WO99/51639 publication claims the use of an integrin subunit α10 or binding entities capable of specifically binding to integrin subunit α10 as markers of cells expressing integrin subunit α10, whereby these cells are chondrocytes, smooth muscle cells, endothelial cells, osteoblasts and fibroblasts (see claims 28, 34, 43, and 60). The cells disclosed in WO99/51639 as expressing integrin subunit α10 do not include stem cells, let alone MSCs. The rejection of the claims under 35 U.S.C. § 102(b) erroneously equates osteoblasts with mesenchymal stem cells (MSC). *Office Action, page 5.* However, an osteoblast is a differentiated, specialized cell type that is destined to its role in bone formation and bone maintenance. To the contrary, a stem cell is an undifferentiated, multipotent cell type that has the potential to give rise to cells of multiple different lineages. As soon as a stem cell commits to a specific lineage or

differentiation pathway, it seizes to be a stem cell. This is illustrated in Figure 1 of the instant application.

Furthermore, the Office cites WO99/51639 for the disclosure that integrin subunit α10 is expressed mostly in cartilage but is also found in perichondrium, periosteum, ossification groove of Ranvier, in fascia surrounding tendon and skeletal muscle and in the tendon-like structures in the heart valves. *Office Action, page 5.* However, this disclosure does not provide any evidence that integrin subunit α10 is expressed in stem cells such as MSCs. In addition, the word "stem cell" is used in WO99/51639 only once, referring to embryonic stem cells in the context of discussing a sequence motif that is present in most integrin α subunits but not in integrin α10 (see page 15, lines 5 to 20).

The rejection under 35 U.S.C. § 102(b) also cites claims 51 and 52 of WO99/51639. *Office Action, page 5.* These claims relate to the use of poly- or oligo-nucleotides for the detection of DNA or RNA encoding integrin α10. In contrast to the claims of the instant application, these claims do not relate to the use of integrin α10 or integrin α10 and integrin α11 protein chains expressed intracellularly or on the cell surface as a marker. Hence, these claims can not contribute to a showing of anticipation.

For all the above reasons, Applicant respectfully submits that WO99/51639 does not anticipate any of the claims currently under consideration and hence requests that the rejections under 35 U.S.C. § 102(b) be withdrawn.

Conclusions

In view of the foregoing amendments and remarks, Applicant respectfully requests reconsideration and reexamination of this application and the timely allowance of the pending claims. If the Examiner believes a telephone conference would be useful in resolving any outstanding issues, the Examiner is invited to call the undersigned at (202) 408-4173.

Please grant any extensions of time required to enter this response and charge any additional required fees to our deposit account 06-0916.

Respectfully submitted,

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Dated: August 7, 2007

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Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2

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Abstract

Bone marrow stromal cells, obtained from postnatal bone marrow, contain progenitors able to differentiate into several mesenchymal lineages. Their use in gene and cell therapy requires their in vitro expansion and calls for the investigation of the culture conditions required to preserve these cells as a stem compartment with high differentiative potential during their life span. Here we report that fibroblast growth factor 2 (FGF-2)-supplemented bone marrow stromal cell primary cultures display an early increase in telomere size followed by a gradual decrease, whereas in control cultures telomere length steadily decreases with increasing population doublings. Together with clonogenic culture conditions, FGF-2 supplementation prolongs the life span of bone marrow stromal cells to more than 70 doublings and maintains their differentiation potential until 50 doublings. These results suggest that FGF-2 in vitro selects for the survival of a particular subset of cells enriched in pluripotent mesenchymal precursors and is useful in obtaining a large number of cells with preserved differentiation potential for mesenchymal tissue repair.

Keywords: Bone marrow stromal cells; Osteoprogenitors; Telomeres; Fibroblast growth factor 2; Tissue engineering

Introduction

Bone marrow stromal cells (BMSC) are fibroblast-like cells that, unlike hematopoietic cells, can be operationally defined by their ability to adhere to plastic surfaces. The first compelling evidence for their differentiation potential derives from transplantation studies in animal models [1]. Indeed, under appropriate conditions BMSC generate in vivo a complete bone structure that includes all major skeletal tissues such as bone, cartilage, and myelosupportive stroma [2]. BMSC are a heterogeneous population that includes committed and pluripotent cells. The evidence for this is the fact that we can isolate from BMSC osteogenic progenitor cells as well as progenitor cells that have bi-, tri- [3], and tetra [4]- lineage differentiation potential. In addition to being multipotential, BMSC are capable of extended proliferation in culture. Therefore, when obtained from relatively small samples, they can be rapidly expanded, efficiently transduced with gene transfer vectors, and transplanted, while still maintaining their differentiation potential [5]. For these reasons BMSC represent a suitable and promising source of autologous cells for both cell therapy and gene therapy of mesenchymal tissues.

Concerns regarding their clinical exploitation can, however, be raised. Indeed, it has been observed that after BMSC reach the first confluence in vitro, their proliferation rate is reduced and it is associated with loss of multipotentiality [6–8]. Furthermore, upon in vitro expansion, in vivo bone-forming efficiency of BMSC is dramatically reduced

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in comparison with fresh bone marrow [8]. This loss of in vivo differentiation potential after in vitro expansion limits drastically the potential use of BMSC for therapeutic purposes.

We have previously reported that the addition of basic fibroblast growth factor (FGF-2) to primary cultures of BMSC helps to maintain their osteogenic potential [9]. Here we show that this effect is associated with a longer telomere size of the cultured cells. Since we have previously found no detectable telomerase activity in expanded BMSC [10], these data indicate that FGF-2 selects a subset of progenitor cells with longer telomeres. When grown both under low-density conditions and in the presence of FGF-2, these cells have an extended life span in vitro of about 70 doublings and they retain their differentiation potential for more than 50 doublings. Thus, FGF-2 enriches BMSC primary cultures for early mesenchymal progenitor cells.

Materials and methods

Cell cultures

Stromal cells were obtained from iliac crest marrow aspirates from healthy donors for bone marrow transplantation (BMT) procedures carried out in S. Martino Hospital and G. Gaslini Pediatric Hospital, both in Genoa (Italy). Donor age ranged between 3 and 49 years and donors were all white Caucasians. Informed consent was obtained from all donors and all procedures were approved by institutional ethical committees. BMSC cultures were performed essentially as previously described [9]. Briefly, mononuclear cells (MNC) were counted, plated at 2–5 × 10^6 MNC/100-mm dish in Coon's modified Ham's F-12 medium supplemented with 10% fetal calf serum (FCS), and half of the plates were cultured in the presence of 1 ng/ml human recombinant FGF-2 (Austral Biologicals, San Ramon, CA, USA). The medium was changed after 3 days and then twice a week.

To evaluate CFU-f frequency (CFU-f assay), 100-μl volumes of the original marrow suspension were plated in 100-mm dishes. The medium was changed after 3 days and then twice a week. After 2 weeks of primary culture, cells were washed with phosphate-buffered saline, pH 7.2 (PBS), fixed with 3.7% formaldehyde in PBS, stained with 1% methylene blue in borate buffer (10 mM, pH 8.8) for 30 min, and washed with distilled water, and the colonies were counted. All determinations were performed in duplicate on bone marrow samples obtained from three different donors and expressed as mean values. CFU-f frequency in the fresh marrow sample was used to calculate the fold expansion and population doublings of first-confluence cultures.

When dishes reached confluence, BMSC were detached with 0.05% trypsin/0.01% EDTA and counted for the population doubling determination. Aliquots of the pooled cells were used for DNA extraction and in vitro differentiation, or replated for life span determination.

Telomerase activity detection and telomere restriction fragment (TRF) length measurement

Telomerase activity was detected as previously described [10]. High-molecular-weight genomic DNA was extracted from BMSC cultures obtained from three different donors with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and measurement of telomere restriction fragment length was performed by Southern blot analysis as previously reported [10,11].

Immunofluorescence staining

To evaluate human telomerase reverse transcriptase (hTERT) expression, 100-μl volumes of the original marrow suspension were plated in 60-mm dishes and cultured as described above both in the presence and in the absence of FGF-2. After 3, 5, 7, and 14 days of primary culture, cells were washed twice in PBS and fixed for 3 min in methanol. Immunofluorescence staining was performed essentially as described by Martin-Rivera et al. [12]. Briefly, after two more washes in PBS, cells were blocked with goat serum and incubated for 1 h at 37°C with a 1/1000th dilution of the telomerase-specific k-370 antibody (Calbiochem–Novabiochem Corp., San Diego, CA, USA). Cells were washed in PBS, and incubated for 1 h with a 1/100th dilution of rhodamine-conjugated anti-rabbit immunoglobulin (Jackson Immunoresearch Laboratories, Inc., West Grove, PA, USA). Nuclei were counterstained with Hoechst 33258. Human HeLa cells were seeded in chamber slides (Becton Dickinson, Franklin Lakes, NJ, USA) and stained as described above as a positive control.

Detection of hTERT was performed on three BMSC cultures obtained from different donors. For each culture the percentage of positive cells was calculated by counting at least 500 cells in three different fields.

In vitro chondrogenic differentiation

Chondrogenic differentiation was performed according to the procedures described by Johnstone and co-workers with minor modifications [13,14]. Briefly, 2.5 × 10^5 cells were pelleted in a 15-ml polypropylene conical tube and were cultured in Coon's modified Ham's F-12 medium supplemented with 10% FCS for 24 h. Culture medium was then replaced with serum-free Coon's modified Ham's F-12 medium supplemented as previously described [14]. Cell pellets were incubated at 37°C, 5% CO_2 and fed every 2 days with fresh medium. At Day 15, cell pellets were fixed with 3.7% formaldehyde in PBS, paraffin embedded, stained with toluidine blue, and analyzed using a Zeiss Axiophot microscope.

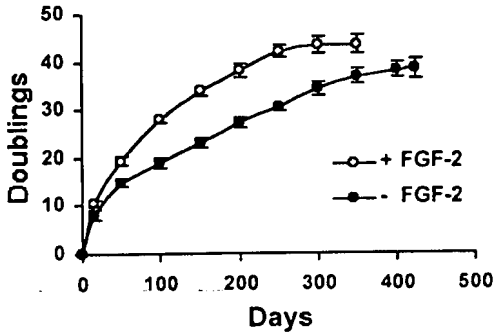

Fig. 1. Life span of BMSC under conditions of high cell density. For life span determination BMSC from donors (41, 11, and 3 years old) were used. When plates reached the first confluence, 100-mm Petri dishes were replated with 5×10^5 cells. BMSC were cultured with or without FGF-2 supplementation until no further proliferation was detected and growth kinetics was determined. Cultures reached the first confluence in about 3 weeks. On replating, BMSC slow their proliferation, regardless of factor supplementation. FGF-2 maintains a higher proliferation rate, but does not significantly affect the life span of treated cultures. Cumulative cell doublings (Db) were plotted against time in culture.

Results

Life span, differentiation, and telomere dynamics under conditions of high cell density

First confluence was reached in about 3 weeks with 12–15 population doublings and subsequently BMSC displayed a slower proliferation rate, which was maintained for the remainder of the life span (Fig. 1). When comparing the curves obtained from the two populations during semiconfluent expansion it is evident that FGF-2 increased the proliferation rate of BMSC but did not significantly affect their in vitro life span, which is set at about 40–45 population doublings (Fig. 1). Concomitant with the slowing of growth rate, BMSC morphology varied as a function of replicative age from the initial spindle shape to a more flattened square or star shape (data not shown). The presence of FGF-2 in the culture medium delayed only the change in cell morphology, which still occurred at increasing cell doublings.

FGF-2 supplementation critically affects the maintenance of chondrogenic potential during expansion. Indeed cartilage was clearly formed only in the cultures that were previously treated with FGF-2, but never in the absence of the factor (Table 1). However, in the FGF-2-supplemented cultures a loss of differentiation potential was observed in parallel with the slowing of proliferation rate. Chondrogenic potential, as assessed by the in vitro assay, was maintained up to the third passage in culture, corresponding to about 21–25 population doublings (Table 1).

To monitor the maintenance of proliferative potential during expansion, we assayed telomere length in primary BMSC grown with or without FGF-2 at every passage along their life span. Telomere restriction fragment (TRF) length measurement was performed on genomic DNA extracted from actively growing cells at increasing population doublings (Fig. 2). To reduce the experimental variability, telomere length has been estimated by running the two samples (with and without FGF-2 supplementation) of each individual side by side as shown in Fig. 2A, where the analysis of a representative culture is reported. Indeed, it has been previously demonstrated that by using this approach the difference between duplicate sample is never higher than 321 bp [11].

In the absence of FGF-2, the expected gradual decrease in telomere size was observed (Fig. 2B). The TRF length decreased by 63 ± 12 bp per cell generation up to 35 doublings. Unexpectedly, however, in the presence of FGF-2 we observed a biphasic pattern, with a transient increase in the population mean telomere size after the first passage, followed by a gradual shortening with a rate similar to that of cultures without FGF-2 (Fig. 2B). Such a pattern in telomere kinetics was reproduced in all the cultures analyzed (Fig. 2C).

Condition switch

Since the early mean TRF length increase was not maintained in the following passages and BMSC do not express telomerase in vitro [10], we tested the hypothesis that FGF-2 might be selecting a population of earlier progenitors rather than directly cause telomere elongation. Fresh bone marrow was plated either with or without FGF-2 supplementation. After increasing periods of time (2 hours, 3 days, 7 days) dishes were washed with PBS and culture conditions were switched. After 14 days of culture, colony number was counted as per the CFU-f assay (see Materials and Methods). Three independent experiments were performed and the data are given in Table 2. We found that in all experimental sets FGF-2 supplementation reduced the colony number by about 30% (Table 2). It should be noted that

Table 1
Chondrogenic differentiation[a]

High-density cultures			Low-density cultures		
Db	+FGF-2	−FGF-2	Db	+ FGF-2	−FGF-2
12–15	+	−	24–27	+	+
17–20	+	−	35–38	+	−
21–25	+	−	48–50	+	−
>25	−	−	>55	−	−

[a] BMSC obtained from three donors of different ages (49 to 3 years old) were used for these experiments. BMSC were cultured in the presence or absence of FGF-2 either under high or low-cell-density conditions and differentiation potential was determined at increasing population doublings. Cartilage formation was considered negative when either sporadic or no chondrocytic lacunae were detected, whereas it was scored as positive in the presence of a clearly detectable cartilaginous metachromatic matrix with chondrocytes embedded in lacunae. Duplicate pellets were differentiated for each sample. At least three histological sections of each pellet were examined and scored (+) positive or (−) negative. Db, population doublings.

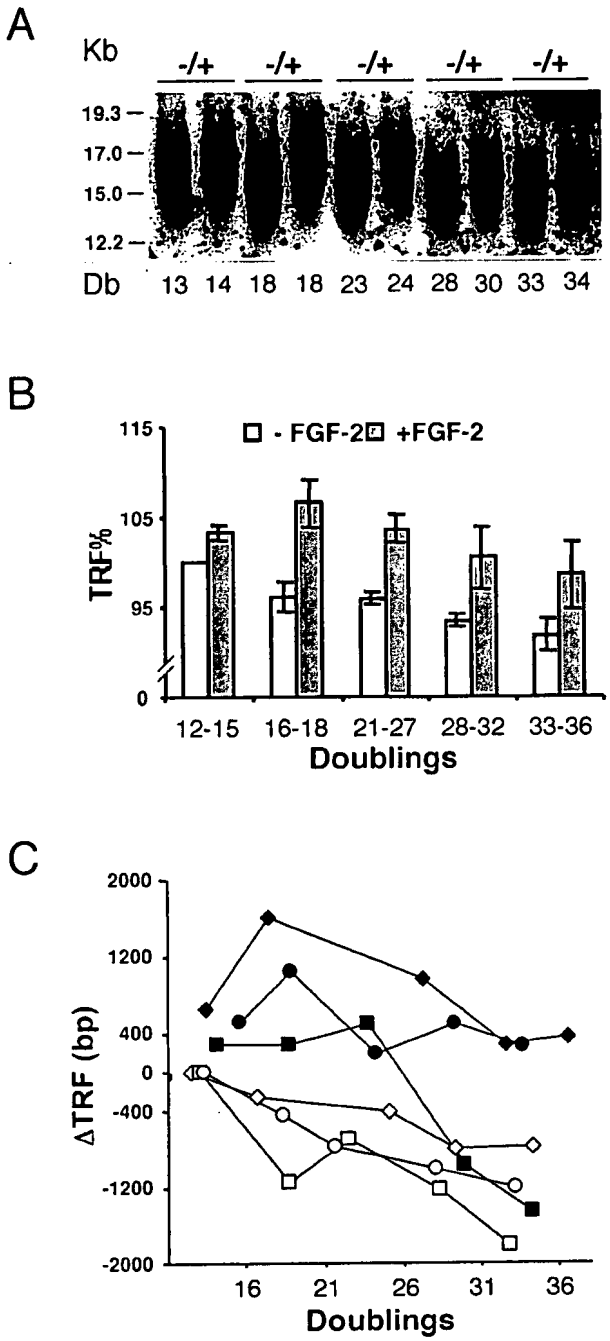

Fig. 2. Telomere dynamics of cultured BMSC. BMSC obtained from three donors of different ages (41, 11, and 3 years old) were cultured both with (+) and without (−) FGF-2 supplementation and telomere length was determined at different population doublings (Db). Genomic DNA was extracted, digested with *Bam*HI, and filter hybridized to the TelBam 8 probe. (A) Southern blot analysis of TRF length of a representative culture. (B) Comparison of the relative TRF length (TRF%) among cultures grown with and without FGF-2. In the absence of FGF-2 there was a gradual decrease in telomere size, whereas in the presence of FGF-2 we observed a biphasic pattern, with an early increase in population mean telomere size, followed by a gradual decrease. (C) Differences in TRF length (ΔTRF) of the three cultures both in the presence (filled symbols) and in the absence (empty symbols) of FGF-2 at increasing population doublings are shown by individual lines.

even 2 h of exposure to FGF-2 was sufficient to inhibit the growth of about 10.5% of the colonies. Interestingly, late exposure to FGF-2 still prevented the development of some colonies, as observed in samples supplemented with FGF-2 starting from the third and seventh days of culture. Samples continuously exposed to FGF-2 always displayed the lowest colony count.

Life span and differentiation kinetics under clonogenic conditions

If the cells selected by FGF-2 are a population with a longer starting telomere length, they should have a significantly longer life span and possibly maintain their differentiation potential longer than unselected BMSC. To assess the proliferative potential of the longest-lived subset of progenitors in primary BMSC, after the first passage, cells were plated under clonogenic conditions (20 cells/cm^2) and passaged every 2 weeks, never allowing the cultures to reach confluence. The procedure was repeated until no further formation of colonies occurred.

For this purpose three different primary cultures were used. BMSC at low density underwent rapid proliferation with a stable growth rate during the whole expansion (Fig. 3). Notably, under clonogenic conditions, FGF-2 caused a significant increase in BMSC life span up to 70 cell doublings, compared with only about 50 without it. Growth rate was always much faster than in semiconfluent cultures, and again FGF-2 caused clonogenic BMSC to proliferate at a constant faster rate. However, BMSC gradually lost their ability to generate colonies (Figs. 4A–D) and smaller colonies were formed at increasing population doublings (Figs. 4E–H). Moreover, late-passage colonies were composed of broad flattened cells and tended to be less confluent than the earlier, larger colonies (Figs. 4I–L).

We also studied the differentiation potential of BMSC

Table 2
Colony number after culture condition switch[a]

	FCS	FGF-2	FCS to FGF-2	FGF-2 to FCS
2 h	100% (54 ± 11)	64.8% ± 1.9	69.7% ± 1.3	89.4% ± 0.9
3 days	100% (95 ± 7)	54.6% ± 1.1	63.8% ± 0.8	68.5% ± 0.7
7 days	100% (98 ± 5)	60.7% ± 0.9	67.5% ± 0.9	75.2% ± 0.5

[a] BMSC obtained from three donors of different ages (49, 26, and 16 years old) were used for these experiments. BMSC were grown either with FGF-2 or with FCS only for increasing amounts of time: 2 h, 3 days, or 7 days. Dishes were then washed with PBS and culture conditions were switched (FCS to FGF-2 and FGF-2 to FCS). Control plates were grown either in FCS only (FCS) or with FGF-2 supplementation (FGF-2) for the whole time. After 14 days of culture colonies were counted as per the CFU-f assay. All determinations were performed in duplicate on bone marrow samples obtained from three different donors and expressed as mean values. The mean colony count of plates grown in FCS only is reported ±SE and assumed to be 100%. The mean colony counts observed in the other three conditions are expressed as percentages ±SE.

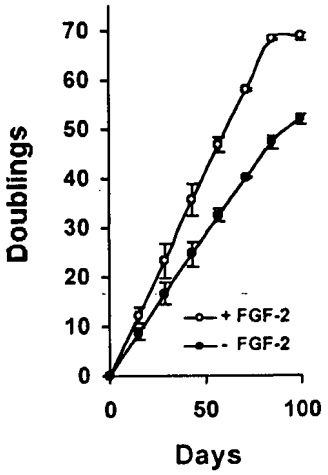

Fig. 3. Life span of BMSC under clonogenic conditions. BMSC obtained from three donors of different ages (49, 26, and 16 years old) were used for these experiments. Two weeks after the initial plating and then regularly every 2 weeks, cells were detached, pooled, and counted, and 10^3 cells were replated in 100-mm dishes, never allowing them to reach confluence. BMSC were cultured with or without FGF-2 supplementation until no proliferation was detected. Both in the presence and in the absence of FGF-2 BMSC proliferate at a steady rate. FGF-2 considerably affected the life span of treated cultures, increasing their growth rate and allowing proliferation up to 72 population doublings, whereas nonsupplemented culture stopped growing after 51 population doublings.

expanded under conditions of low cell density. Aliquots of cells expanded either with or without FGF-2 were tested for in vitro cartilage formation, as we previously showed that this ability reliably predicts the osteogenic differentiation of the population [14]. Under clonogenic conditions, BMSC without FGF-2 retained their ability to differentiate into cartilage for about 27 doublings, never doing so when grown to semiconfluence (Table 1). In addition, FGF-2 significantly extended the maintenance of such potential, so that cells were still capable of chondrogenic differentiation up to 50 population doublings (Table 1).

The loss of TRF length after the initial increase suggests that FGF-2 is not capable of maintaining the proliferative potential of BMSC in vitro. Using the telomeric repeat amplification protocol (TRAP) assay, we found no detectable levels of telomerase activity in BMSC primary cultures at both early and late passages, even when derived from the younger donor analyzed (Fig. 5). To verify whether the subpopulation of FGF-2-selected progenitors might express telomerase early in the culture and subsequently lose it during expansion, we performed a single-cell analysis of hTERT expression in colonies formed respectively at 3, 5, 7, and 14 days after bone marrow seeding by immunofluorescence staining with telomerase-specific k-370 antibody. BMSC plated either in the presence or in the absence of

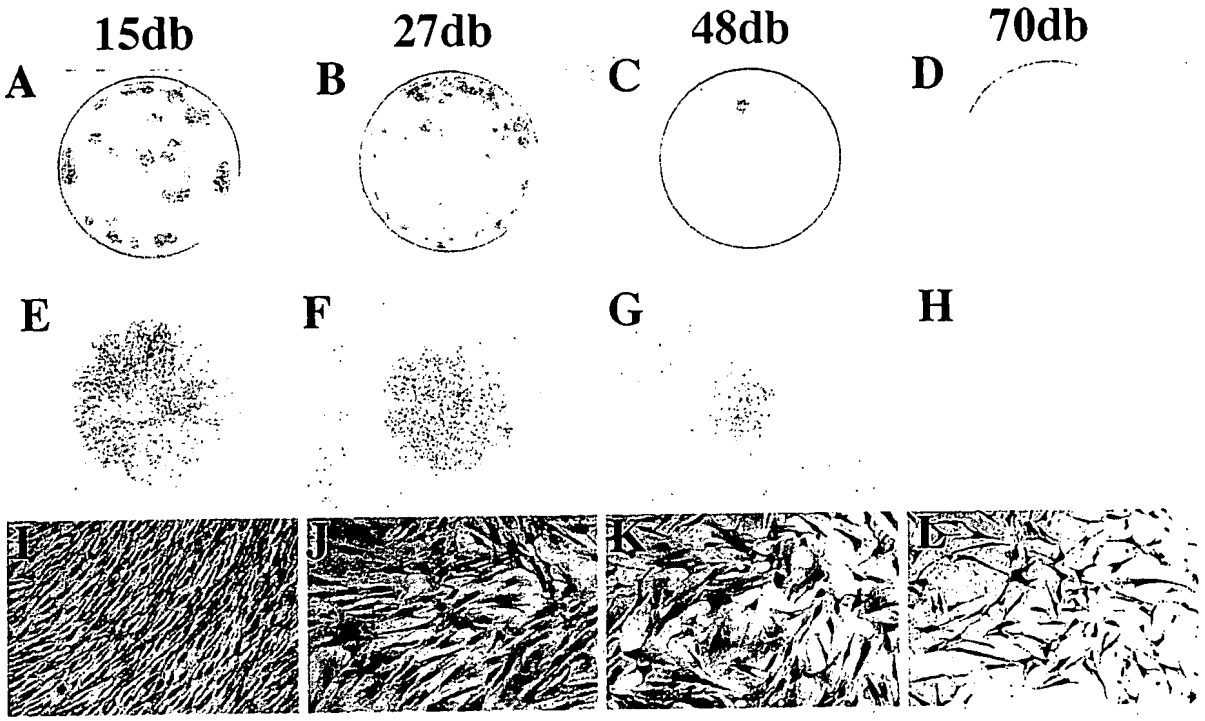

Fig. 4. Colony and cell morphology under clonogenic conditions. BMSC obtained from three donors of different ages (49, 26, and 16 years old) were used for these experiments. Clonogenic fraction was determined at increasing population doublings by plating 10^3 cells per 100-mm dish which was kept in culture for 2 weeks and then processed as per the CFU-f assay. Colonies were counted and the results are expressed as the mean value of two plates. Colony number and morphology were monitored under clonogenic conditions, either with or without FGF-2 supplementation, at increasing cell doublings as indicated. The number of colonies obtained on replating progressively decreased: A, B, C, and D. Also, colony size is affected during in vitro expansion: E, F, G, and H. Cells progressively were less confluent and at the same time assumed a flattened phenotype: I, J, K, and L. Magnification: (E, F, G, H) 2.5×; (I, J, K, L) 10×.

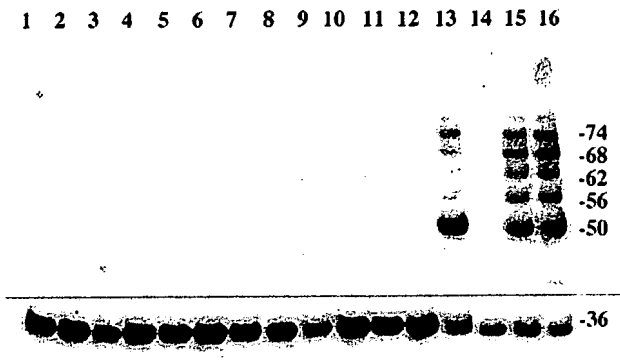

Fig. 5. Telomerase activity in BMSC. Telomerase expression was never detected in samples of proliferating human BMSC from three different primary cultures obtained from donors 41, 11, and 3 years old. Corresponding population doublings are for + FGF: lanes 1, 2 = 15, lanes 3, 4 = 18, lanes 5, 6 = 32; − FGF: lanes 7, 8 = 12; lanes 9, 10 = 16; lanes 11, 12 = 28. Five thousand cell equivalents of the experimental samples were assayed without (lanes 1, 3, 5, 7, 9) and with inactivation by heat treatment (lanes 2, 4, 6, 8, 10, 12). Lane 13: 500 cell equivalents of telomerase-positive control cells. Lane 14: CHAPS lysis buffer negative control. Lanes 15 and 16: quantitation control, with 0.1 and 0.2 nmol of TSR8 standard template. Numbers on the right-hand side indicate the size in base pairs of the first five bands of the telomerase ladder and of the PCR internal control (36 bp).

FGF-2 did not display any specific signal, confirming that possibly hTERT protein levels in these cells are undetectable even at the CFU-f stage (data not shown).

Discussion

The use of BMSC in gene and cell therapy requires their in vitro expansion and culture conditions that preserve their differentiation and proliferative potential. Recently, the isolation of cells capable of differentiating into cells of the three germ layers has been performed by growing adherent cells obtained from postnatal bone marrow on fibronectin in the presence of different growth factors [15,16]. To obtain large numbers of osteoprogenitors for cell transplantation, the effects of growth factors on proliferation and differentiation of BMSC are of great interest and have been investigated by several groups (9,17–24). It has been demonstrated that FGF-2 supplementation of BMSC cultures increases the frequency of tripotential progenitors, capable of osteogenic, chondrogenic, and adipogenic differentiation at a clonal level [3]. Further, it promotes the proliferation and maintains the osteogenicity of this heterogeneous cell population, thus allowing the in vitro expansion necessary for some clinical applications such as bone reconstruction [9–25]. In this report we have found that FGF-2 supplementation of BMSC primary cultures selects a subpopulation of earlier progenitors with a significantly longer life span and that maintain their differentiation potential for more than 50 population doublings in vitro.

The gradual loss of telomeric repeats has been regarded as a "mitotic clock" leading to cell cycle arrest or cellular senescence [26]. Telomerase, a ribonucleoprotein enzyme that prevents telomere shortening by de novo synthesizing telomere repeats, operates mainly in cells capable of unlimited proliferation such as germ-line cells, embryonic stem cells, immortal cell lines, and cancer cells [27–31]. In humans, telomeres shorten both with cell senescence in vitro and with aging in vivo in normal somatic cells that do not express telomerase [26,32–34]. Notable exceptions are hematopoietic and other stem cells that express low levels of telomerase [34] which in most cases are unable to prevent telomere shortening in vivo [11,35]. We have previously demonstrated that BMSC do not display telomerase activity and undergo replicative aging with extended proliferation in culture [10]. Recently, two different studies demonstrated that expression of ectopic telomerase increases the life span of BMSC without affecting the proliferation rate; telomerase-transduced BMSC display also an increased bone formation capability [36,37]. In this study we have investigated the biological effects of FGF-2 supplementation on telomere dynamics and in vitro life span of proliferating BMSC.

Without FGF-2, telomere length decreased with increasing population doublings, as expected for normal cells that do not display telomerase activity, whereas FGF-2-supplemented cultures showed an early increase in telomere size followed by a gradual decrease. This effect must start immediately after plating, as the FGF-2-treated cells already display a longer mean TRF when reaching the first confluence compared with the same marrow cultured without factor supplementation. Since we found no telomerase expression, either at the stage of primary colonies, as detected by immunofluorescence, or during the entire culture life span [10], we can rule out that this result reflects a physical increase in telomere length. Our results suggest instead that, in the early stages of the culture, FGF-2 selects for a pre-existing population of cells with longer telomeres.

The results shown in Table 2 indicate that primary BMSC are readily responsive to FGF-2 and its effects are inhibition of colony formation in a subset of BMSC and stimulation of proliferation in another subset of cells. Interestingly, this effect seems extremely rapid, as the exposure of freshly plated marrow to FGF-2 for only 2 h already reduced colony formation by 10% (Table 2). In osteoblasts and BMSC, FGF-2 stimulates proliferation and reduces markers of osteogenic differentiation such as ALP [9,38]. In osteoblasts, however, the response to FGF-2 is bimodal, as immature cells increase proliferation, whereas differentiating osteoblasts actually undergo apoptosis [38]. Our results suggest that the initial increase in telomere length is due to negative selection by FGF-2 on the subset of BMSC already committed to the osteogenic lineage and proliferative stimulation of more immature progenitors with longer telomeres. Whether apoptosis is involved in this effect is unclear and further experiments are needed to elucidate this point. In addition we observed that in our cultures telomere-directed senescence is not related to absolute telomere

length, but to the extent of telomere shortening. In fact, growth arrest in FGF-2-treated cultures undergoing telomere shortening occurred when telomeres were still longer than in nontreated cultures (Figs. 2B, C). From our data we cannot rule out the existence among FGF-2-selected BMSC of a small population with shorter telomeres as well as of a population with longer telomeres in unselected BMSC. It might be speculated that the two populations (shorter and longer telomere BMSC) affect each other according to the relative abundance. In particular, senescent cells might inhibit the proliferative capacity of cells with longer telomeres. Additional experiments are required to address this point.

Consistent with previous studies we found that BMSC have a limited life span in culture [24]. FGF-2 reproducibly increased the proliferation rate in standard BMSC cultures, although it did not significantly affect the life span, as growth arrest occurred after 40–45 doublings regardless of factor supplementation. Low cell density conditions have been demonstrated to strongly increase the expandability of BMSC in culture [39,40]. Moreover, colonies formed in the CFU-f assay derive from a single cell and consequently their count gives an estimation of the number of early mesenchymal precursors capable of significant expansion in a sample of bone marrow [7,41,42]. Under low cell density conditions committed and slow-proliferating cells are selected against and only the clonogenic subset of cells is maintained in culture. Therefore, at low density the population with the highest proliferative potential determines the life span of the whole culture. When cultured under clonogenic conditions, the population selected by FGF-2 proliferates at a constant rate for more than 70 doublings. Such a life span is longer than that displayed by normal somatic cells (fewer than 50 doublings [43]) and is more suggestive of an earlier progenitor. Even more remarkably, FGF-2-supplemented clonogenic cultures retained their differentiation potential for at least 50 doublings, i.e., twice as long as without the factor. Chondrogenesis in micromass pellet culture has been regarded as a rapid predictive assay to assess BMSC multipotentiality. Indeed the in vitro chondrogenic potential of BMSC is directly correlated to their ability to form bone after in vivo transplantation [14]. Nevertheless, FGF-2 does not appear to be able to maintain such progenitors indefinitely, as with continued subculturing the clonogenic fraction decreased and cells approached senescence, losing their ability to differentiate, or alternatively became committed to a specific differentiation pathway, as suggested by the observation of spontaneous differentiation into osteoblasts after extended expansion [6–8].

The results presented in this study demonstrate that FGF-2 supplementation of primary BMSC in vitro can select for the survival of a particular subset of cells enriched in pluripotent mesenchymal precursors and, in conjunction with clonogenic culture conditions, prolong their life span to more than 70 doublings and maintain their differentiation potential until 50 doublings. Even though FGF-2 is not sufficient by itself to maintain a steady number of progenitors along the expansion, our observations show that FGF-2 is a relevant component of new culture conditions to exploit the full potential of BMSC for tissue engineering approaches.

Acknowledgments

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Retention of Multilineage Differentiation Potential of Mesenchymal Cells during Proliferation in Response to FGF

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Mesenchymal stem cells (MSC) that can differentiate to various connective tissue cells may be useful for autologous cell transplantation to defects of bone, cartilage, and tendon, if MSC can be expanded *in vitro*. However, a short life span of MSC and a reduction in their differentiation potential in culture have limited their clinical application. The purpose of this study is to identify a growth factor(s) involved in self-renewal of MSC and the maintenance of their multilineage differentiation potential. Fibroblast growth factor-2 (FGF-2) markedly increased the growth rate and the life span of rabbit, canine, and human bone marrow MSC in monolayer cultures. This effect of FGF-2 was more prominent in low-density cultures than in high-density cultures. In addition, all MSC expanded *in vitro* with FGF-2, but not without FGF-2, differentiated to chondrocytes in pellet cultures. The FGF(+) MSC also retained the osteogenic and adipogenic potential throughout many mitotic divisions. These findings suggest that FGFs play a crucial role in self-renewal of MSC. © 2001 Academic Press

Key Words: **mesenchymal stem cell; FGF; multilineage differentiation potential; life span.**

Embryonic tissues contain mesenchymal cells that differentiate to osteoblasts, chondrocytes, adipocytes, hematopoietic cells, endothelial cells, and muscle cells. Adult bone marrow, as well as the periosteum, adipose tissue and peripheral blood, also contain "mesenchymal stem cells (MSC)" (1–3). The relationship between embryonic and adult MSC is unknown. However, bone marrow-derived MSC can differentiate to osteoblasts, chondrocytes, tenocytes, adipocytes, muscle cells or nerve cells *in vitro* and/or *in vivo* (1–6). MSC can easily be obtained repeatedly by bone marrow aspiration. Thus transplantation of bone marrow MSC may provide a new method for treatment of osteoporosis, arthritis, periodontal diseases, intrinsic muscular dystrophies, cardiac diseases, and degenerative nerve diseases (5). However, isolation of marrow aspirates in great volume causes damage and pain, and it is difficult to isolate from the bone marrow 10^7–10^8 MSC that are required for regeneration of large injured tissues. Thus the expansion of MSC *in vitro* is a prerequisite for autologous cell transplantation. In other words, identification of growth factors that stimulate the proliferation of MSC and support their multilineage differentiation potential is a critical step towards the clinical application of MSC.

Previous studies have shown that FGF is a potent mitogen for some connective tissue cells including osteoblasts and chondrocytes (8). Furthermore, chondrocytes grown with FGF-2 *in vitro* but not without FGF-2 maintained the capability for the phenotypic expression after several mitotic divisions, even though direct addition of FGF-2 in confluent cultures markedly suppressed the phenotypic expression (8). These findings prompted us to examine the effect of FGF-2 on the proliferation and differentiation of MSC. The results showed that FGF-2 is a potent mitogen for MSC, and that incubation with FGF-2 maintains the multilineage differentiation potential of MSC throughout many mitotic divisions.

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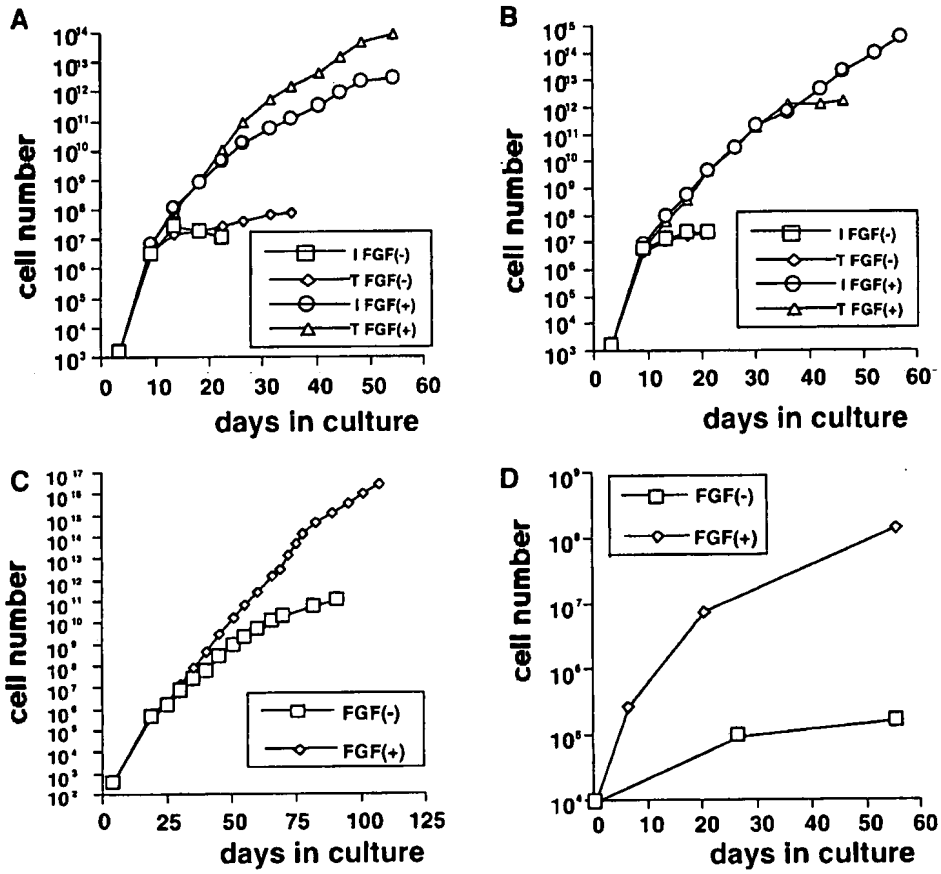

FIG. 1. Culture lifetime of rabbit (A, B) and human MSC (C, D) in monolayer cultures in the presence and absence of FGF-2. MSC were isolated from the ilium (I) or tibia (T) of three rabbits in two independent studies (A, B) or from the ilium of two patients (C, D). MSC used in D were obtained from BioWhittaker Inc. MSC were seeded at 1 (D) or 5×10^3 cells (A–C) per cm^2 and maintained in the medium containing 10% fetal bovine serum (A–C) or human serum (D) in the presence or absence of FGF-2 (1 ng/ml). Passages were performed when the cells were approaching confluence.

MATERIALS AND METHODS

Cell culture. Human bone marrow MSC were obtained from Bio-Whittaker Inc. (secondary cultures, Walkersville, MD) or patents as part of a protocol approved by ethical authorities. Marrow aspirates were also obtained from three 4- or 12-week-old male Japan White rabbits. The cells, including erythrocytes (0.1 ml aliquots of the aspirates), were seeded at 2×10^8 cells per 100-mm tissue culture dish and maintained in 10 ml of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and antibiotics (medium A). Three days after seeding, floating cells were removed and the medium was replaced by flesh medium A. Thereafter attached cells were fed with fresh medium A every three days. Passages were performed when cells were approaching confluence. Cells were seeded at 1 or 5×10^3 cells per cm^2 in 100-mm dishes.

Chondrogenic, osteogenic or adipogenic conversion of MSC was determined according to the procedures reported by Pittenger *et al.* with some modifications (1). For chondrogenic differentiation, cells were seeded at 2×10^5 cells per 15 ml plastic centrifuge tube, and maintained in 0.5 ml of serum-free α-MEM (high glucose) supplemented with 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenite, 5.33 μg/ml linolate, 1.25 mg/ml bovine serum albumin, 10 ng/ml TGF-β-1, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate (Wako, Tokyo, Japan). The cultures were fed with 0.5 ml of the medium until 4 days after seeding. Thereafter, the cultures were fed with 1 ml of the medium every other day.

For osteogenic differentiation, cells were seeded at 10^4 cells per 9-mm-dish, and maintained for 28 days in DMEM supplemented with 10 μg/ml insulin, 10 mM β-glycerophosphate, 100 nM dexamethasone, and 50 μg/ml ascorbic acid-2-phosphate.

For adipogenic differentiation, cells were seeded at 4×10^4 cells per 9-mm dish and maintained in DMEM containing 10% fetal bovine serum and antibiotics for 3 days. The cells were exposed to DMEM (high glucose) supplemented with 10 μg/ml insulin, 0.2 mM indomethacin, 1 μM dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 10% fetal bovine serum for 25 days. Lipid was stained with oil-red O.

Glycosaminoglycan, alkaline phosphatase activity, and calcium. The glycosaminoglycan content was determined as described previously (9). Alkaline phosphatase activity in pellet cultures was determined by the method of Bessey *et al.* (10). The calcium content of monolayer cultures in 11-mm dishes was determined by the method of Gitelman (11).

RT-PCR. Total RNA was extracted using Isogen (Nippon Gene, Tokyo, Japan). The first-strand cDNA was synthesized from 1 μg of total RNA using the Superscript II RNase H^- reverse transcriptase (Life Technologies, Inc., Rockville, MD). Using the cDNAs as a template, PCR was carried out under the following conditions: denaturation at 94°C for 30 s and primer extension at 65°C for 1.5 min in 25, 30, 30, 30, 25, 30, 30 cycles for type II collagen and type X collagen,

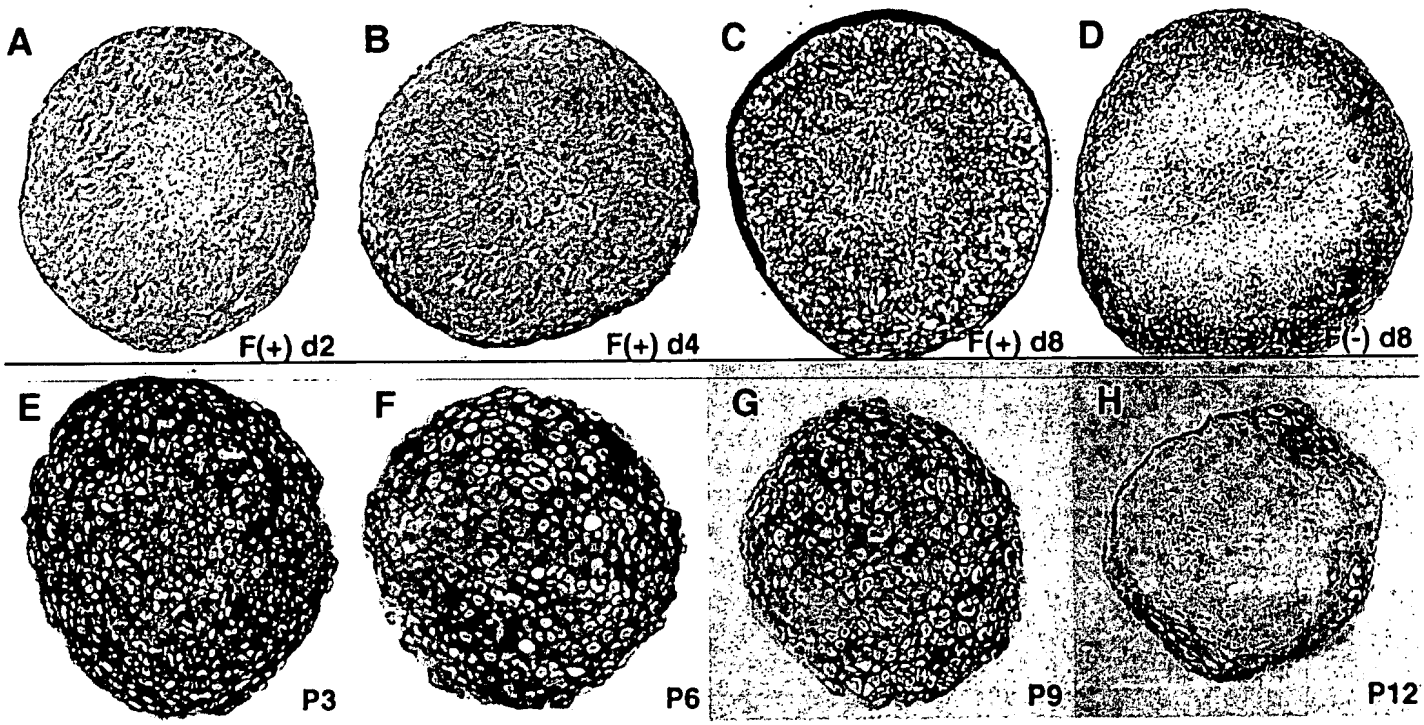

FIG. 2. Chondrogenic potential of MSC expanded *ex vivo* with FGF-2. (A) MSC isolated from 4-week-old rabbits and grown in the presence (A–C) or absence (D) of FGF-2. The MSC obtained from the 3rd passage cultures were transferred into the chondrogenic medium in pellet cultures for 2 (A), 4 (B), and 8 days (C, D). FGF(+) MSC from the 3rd, 6th, 9th, and 12th passage cultures (E–H, respectively) were maintained in pellet cultures for 16 days.

GAPDH, bone sialoprotein, osteopontin, osteocalcin and PPAR-γ2, respectively. Pairs of nucleotides, 5′-CATACCGGTAAGTGGGGC-AAGACTG-3′ and 5′-TGCCCAGTTCAGGTCTCTTA-3′ for rabbit type II collagen, 5′-CCCAACACCAAGACACAGTT-3′ and 5′-ATC-ACCTTTGATGCCTGGCT-3′ for rabbit type X collagen, and 5′-GTC-AAGGCCGAGAATGGGAA-3′ and 5′-GCTTCACCACCTTCTTG-ATG-3′ for rabbit and human GAPDH, 5′-CATTTTGGGAAT-GGCCTGTG-3′ and 5′-ATTGTCTCCTCCGCTGCTGC-3′ for human bone sialoprotein, 5′-CTAGGCATCACCTGTGCCATACC-3′ and 5′-CAGTGACCAGTTCATCAGATTCATC-3′ human osteopontin, 5′-CCACCGAGACACCATGAGAG-3′ and 5′-CCATAGGGCTGGGA-GGTCAG-3′ for human osteocalcin, and 5′-CATTCTGGCCCACCAA-CTT-3′ and 5′-CCTTGCATCCTTCACAAGCA-3′ human PPAR-γ2 were used as primers for RT-PCR. Obtained PCR products were separated on 1% agarose gels, and stained with ethidium bromide.

RESULTS

FGF-2 increased the growth rate and the life span of rabbit and human MSC from the ilium and/or tibia in monolayer cultures in the presence of 10% fetal bovine serum (Fig. 1). FGF-2 had greater effects in low-density cultures (seeding density, 1000 cells/cm^2) than in high-density cultures (5000 cells/cm^2) in all series of studies (data not shown). Similar results were obtained with human alveolar bone MSC and canine ilium bone marrow MSC (data not shown).

To examine the chondrogenic potential, MSC grown with FGF-2 (FGF(+) MSC) were transferred into the chondrogenic medium containing TGF-β and insulin in pellet cultures (Fig. 2A). The chondrogenic medium did not contain FGF-2. Chondrocyte differentiation occurs at a much higher level in pellet cultures than in monolayer cultures (12). In the pellet cultures of FGF(+) MSC, spherical cells (chondrocytes) appeared near the surface of the pellet on day 4 even in the absence of FGF-2 (Fig. 2B). The chondrocytes were surrounded by cartilage-characteristic proteoglycan that stained metachromatically with toluidine blue. On day 8, all cells in FGF(+) MSC cultures (Fig. 2C) became spherical. However, the chondrogenic differentiation was markedly delayed in cultures of FGF(−) MSC (Fig. 2D).

The chondrogenic potential of FGF(+) MSC was examined as a function of the passage number. FGF(+) MSC from the 3rd, 6th, 9th, and 12th passage cultures (Figs. 2E–2H, respectively) were maintained in pellet cultures for 16 days. Almost all FGF(+) MSC from the 3rd through 9th passage cultures reorganized into a cartilage-like tissue by day 16 (Figs. 2D–2G). However, toluidine blue stained cartilage proteoglycan decreased with the increase in the passage number. A few FGF(+) MSC from the 12th passage cultures became chondrocytes (Fig. 2H).

The glycosaminoglycan and alkaline phosphatase levels decreased with the increase in the passage number in the FGF(+) and FGF(−) MSC cultures. How-

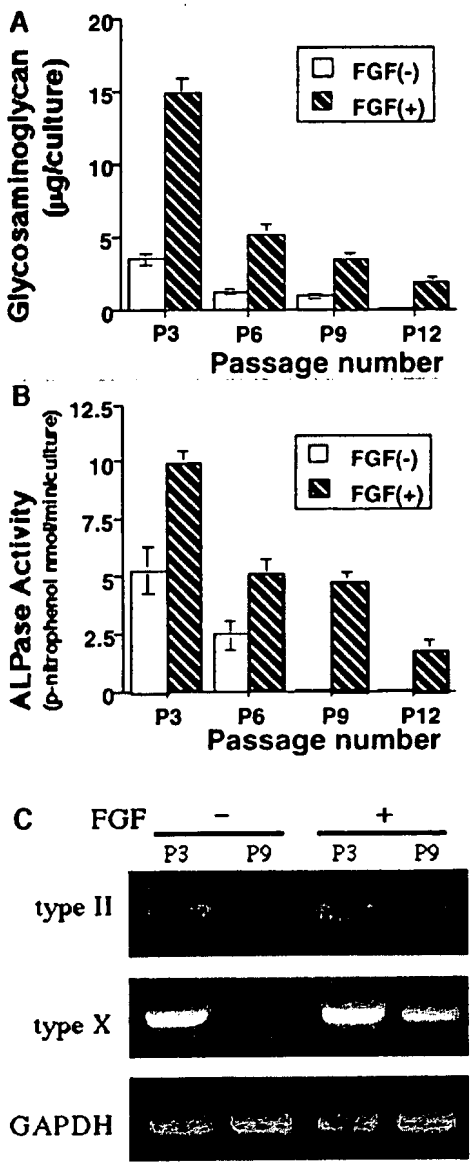

FIG. 3. The levels of glycosaminoglycan, alkaline phosphatase and cartilage-specific gene expression in pellet cultures of FGF(+) and FGF(−) MSC as a function of the passage number. FGF(+) and FGF(−) MSC from the 3rd, 6th, 9th, and 12th passage cultures were maintained in pellet cultures. The glycosaminoglycan content (A), alkaline phosphatase activity (B), and type II and type X collagen mRNA levels (C) were determined 16 days after seeding. Values are averages ± SD for four cultures.

ever, at each passage number, the glycosaminoglycan (Fig. 3A) and alkaline phosphatase levels (Fig. 3B) were much higher in FGF(+) MSC cultures than in FGF(−) MSC cultures. Furthermore, the levels of type II collagen and type X collagen mRNAs, markers for chondrocytes, were higher in FGF(+) MSC from the 9th passage cultures than in FGF(−) MSC from the 9th passage cultures (Fig. 3C).

Next FGF(+) and FGF(−) MSC grown in high- or low-density cultures for many generations were transferred into the osteogenic medium. When MSC were passaged at a high initial cell density (5000 cells/cm^2), FGF(+) and FGF(−) MSC from the 3rd, 6th, and 9th passage cultures showed similar calcium and alkaline phosphatase levels, irrespective of the passage number or the presence or absence of FGF-2 (Figs. 4A and 4B). When MSC were passaged at a low initial cell density (1000 cells/cm^2), FGF(+) MSC showed a higher calcium content than FGF(−) MSC, and the calcium content decreased with the increase in the passage number (Fig. 4C). The expressions of bone sialoprotein, osteopontin and osteocalcin mRNAs in cultures of FGF(+) MSC from the 9th passage cultures were higher than in cultures of FGF(−) MSC from the 9th passage cultures (Fig. 4D). These observations suggest that FGF-2 maintains the osteogenic potential of MSC.

FGF(+) and FGF(−) MSC showed similar adipogenic differentiation, which was estimated by oil-red O staining (Figs. 5A and 5B). The level of PPAR-γ2 mRNA, a marker of adipocyte differentiation, in the FGF(+) and FGF(−) MSC cultures was almost constant, at least until the 9th passage (Fig. 5C).

DISCUSSION

The use of leukemia inhibitory factor (LIF) and feeder cells allowed the proliferation of embryonic stem cells (ES cells) *in vitro*. The availability of the cultured ES cells greatly contributed to developmental biology. Similarly the availability of large amounts of MSC expanded with FGF-2 *in vitro* will facilitate the clinical application of MSC and basic studies on MSC.

The chondrogenic potential of adult MSC markedly decreased with the increase in the passage number, even when the cells were seeded at a high initial cell density. Incubation of MSC with FGF-2 maintained the chondrogenic potential during the expansion of MSC *in vitro*. However, even in the presence of FGF-2, the chondrogenic potential gradually decreased with the passage number. Additional factors seem to be required for the maintenance of the chondrogenic potential. In contrast, the osteogenic potential of MSC was maintained at a high level throughout many mitotic divisions, when MSC were seeded and grown in high-density cultures in the presence of FGF-2. The osteogenic potential decreased when MSC were seeded and grown in low-density cultures in the absence of FGF. The adipogenic potential of MSC was maintained at a high level even in the absence of FGF-2 throughout many mitogenic divisions. These observations suggest that the chondrogenic potential of MSC is unstable throughout mitotic divisions *in vitro*, as compared to the osteogenic and adipogenic potential. FGF-2 markedly suppressed the decline in the chondrogenic/osteogenic potential of MSC, when it enhanced cell prolifera-

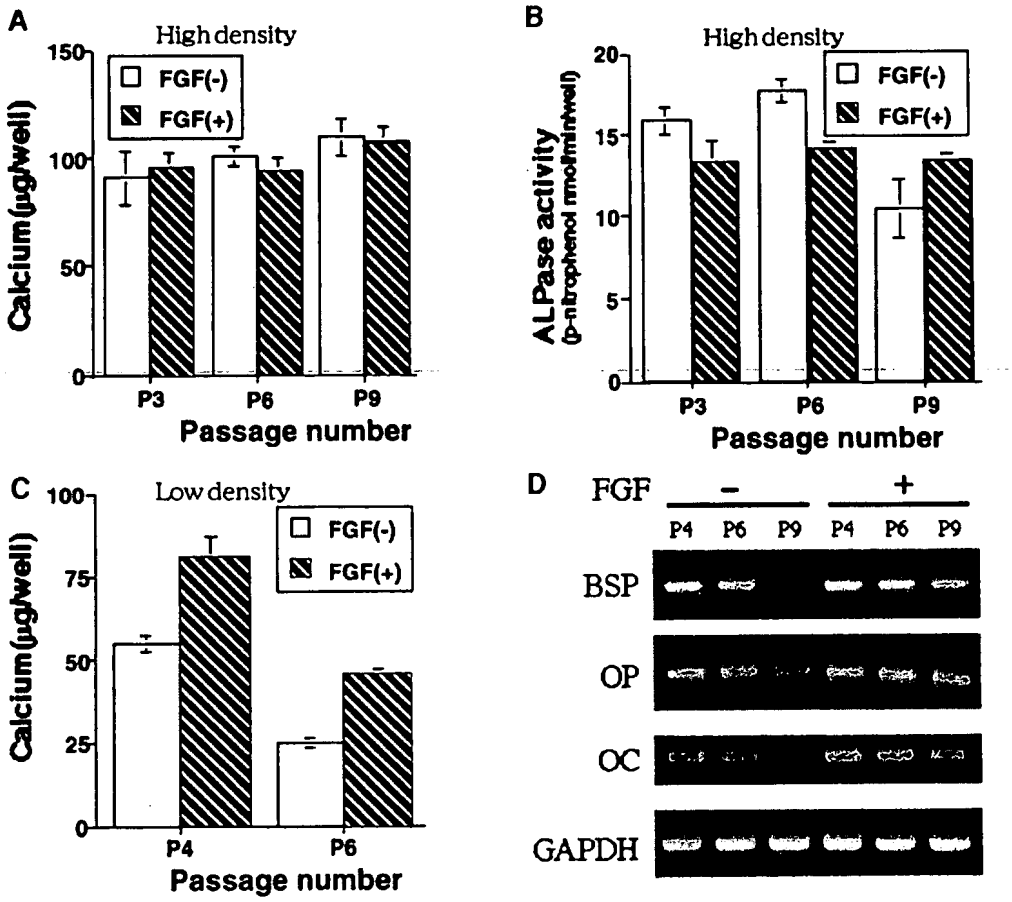

FIG. 4. Osteogenic potential of MSC expanded *ex vivo* with or without FGF-2. Human MSC were seeded at a high (5000 cells/cm^2) (A, B, D) or low (1000 cells/cm^2) density (C). The MSC from the 3rd, 4th, 6th, and 9th passage cultures with or without FGF-2 were transferred into the osteogenic medium for 28 days, and the calcium content (A, C) and alkaline phosphatase activity (B) of the cell-matrix layers were determined. Values are averages ± SD for four cultures. (D) RNA was isolated on day 28. RT-PCR analysis of osteoblast-specific gene expression (bone sialoprotein, osteopontin, and osteocalcin) in cultures of FGF(+) or FGF(−).

tion. The mechanism by which FGF-2 maintains the differentiation potential of MSC is unknown.

Studies with FGF-2-knockout mice have shown that bone mass is smaller in FGF-2-knockout mice than control mice, and that bone marrow mesenchymal cells from FGF-2-knockout mice have poor osteogenic potential *in vitro* (13). These findings, taken together with our findings in the present study, suggest that FGF-2 plays a role in the maintenance of the osteogenic potential of MSC *in vivo*. Since the FGF family has many members (>23), it is difficult to determine which FGF family members play the most critical role in self-renewal of MSC.

Oct-3/4 has been shown to be expressed at high levels in ES and EG (embryonic germ) cells and essential for the maintenance of the undifferentiated state of these cells (14). LIF activates STAT3 via LIF receptors and gp130 to maintain the undifferentiated stage of ES cells (15). The mechanism involved in the maintenance of the undifferentiated state or self-renewal of MSC remains unknown. However, MSC maintained in the presence of FGF may be useful for identification of signaling molecules and transcription factor(s) involved in self-renewal and the multilineage differentiation potential of MSC. We are investigating FGF-inducible genes in MSC using DNA arrays.

In preliminary studies, we transplanted rabbit MSC into 5-mm-diameter full-thickness defects in rabbit knee joints. MSC were obtained from the 3rd passage cultures of 12-week-old rabbits, and expanded *ex vivo* with FGF-2, before transplantation. Nine weeks after transplantation, nontreated defects were not covered with a cartilaginous tissue, although a bone-like tissue was formed. In contrast, MSC-treated defects were completely covered with cartilage, which was indistinguishable from the surrounding original articular cartilage (data not shown). Transplantation of FGF(+) MSC will be useful for tissue regeneration *in vivo*.

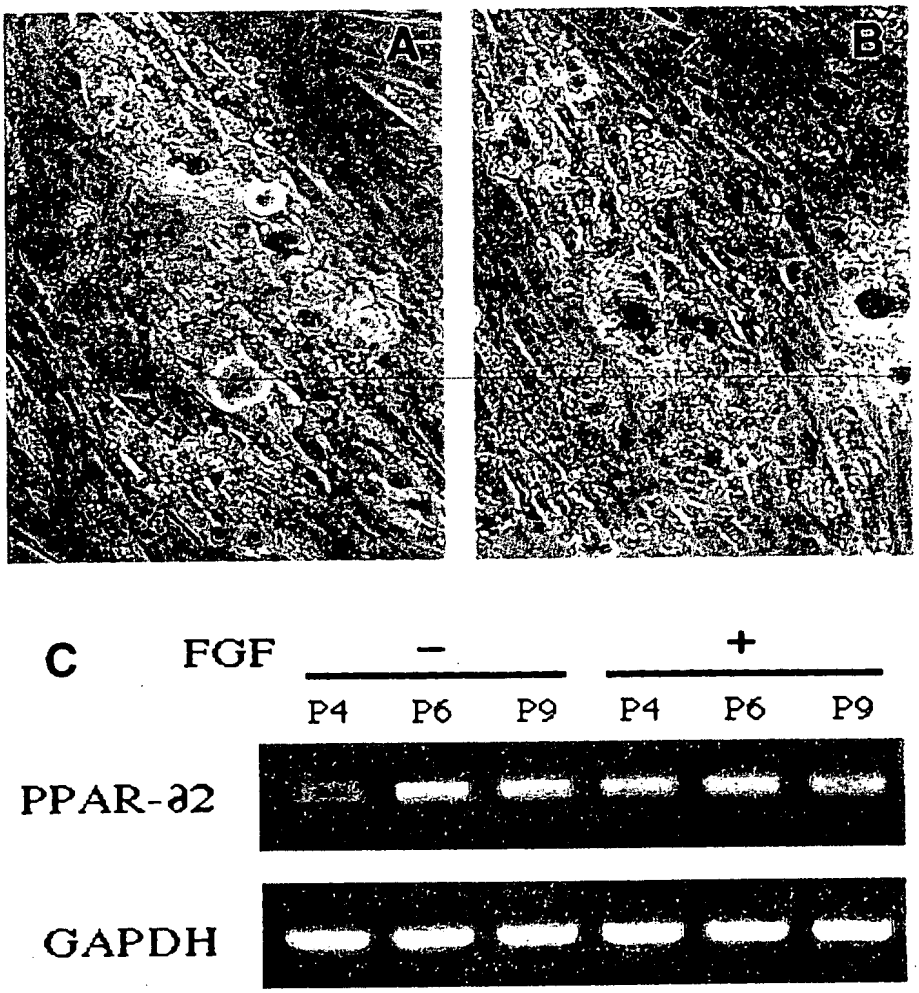

FIG. 5. Adipogenic potential of MSC expanded *ex vivo* without (A) or with FGF-2 (B). Human MSC were seeded at 5000 cells/cm^2. The FGF(+) and FGF(−) MSC obtained from the 6th (A, B) and the 4th, 6th, and 9th passage cultures (C) were maintained under the adipogenic conditions for 28 days. RT-PCR analysis of adipocyte-specific gene expression (PPAR-γ2) in cultures of FGF(+) or FGF(−) MSC on day 28 (C).

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STEM CELLS
TISSUE-SPECIFIC STEM CELLS

Autocrine Fibroblast Growth Factor 2 Signaling Is Critical for Self-Renewal of Human Multipotent Adipose-Derived Stem Cells

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Key Words. Adult stem cells • Self-renewal • Fibroblast growth factor • Long-term expansion • Adipogenesis

ABSTRACT

Adipose tissue-derived stem cells offer tremendous potential for regenerative medicine. However, characterization of their self-renewal ability has not been performed yet, although it is a crucial feature for in vitro expansion of undifferentiated cells and in vivo maintenance of stem cell pools. We have undertaken the identification of molecular events that are involved in in vitro self-renewal of human multipotent adipose-derived stem (hMADS) cells from young donors, by assessing their proliferation rate, their ability to grow at the single-cell level (clonogenicity), and their differentiation potential. As hMADS cells are propagated in culture, cell morphology changes dramatically, concomitantly to a progressive decrease in proliferation, clonogenicity, and differentiation potential. This decrease is associated with a decrease in fibroblast growth factor 2 (FGF2) expression and can be circumvented by chronic treatment with exogenous FGF2. Moreover, analysis of FGF2 secretion revealed that it is exported to hMADS cell surface without being released into the culture medium, suggesting a strictly autocrine loop. Indeed, treatment of FGF2-expressing hMADS cells with PD173074, a specific FGF receptor inhibitor, decreases dramatically their clonogenicity and differentiation potential. Thus, hMADS cells express a functional autocrine FGF loop that allows maintenance of their self-renewal ability in vitro. Finally, inhibition of mitogen-activated protein kinase kinase 1 reduces the clonogenic potential of hMADS cells but does not affect their differentiation potential, indicating that the extracellular signal-related kinases 1/2 signaling pathway is partly involved in FGF2-mediated self-renewal. Together, our data clearly identify the key function of FGF2 in the maintenance of self-renewal of adipose tissue-derived stem cells. STEM CELLS *2006;24: 2412–2419*

INTRODUCTION

The prospective use of stem cells for the restoration of injured or diseased tissues has opened new fields of research. Plasticity is the first requirement for this therapeutic potential. Several studies have demonstrated that this feature is not restricted to embryonic stem cells. A number of stem cells isolated from adult tissues have proven to be multipotent in vitro and have been successfully used for tissue repair in vivo [1, 2]. Much attention has been paid to mesenchymal stem cells from human bone marrow because of their extended plasticity [3, 4]. Within this category, multipotent adult progenitor cells are able, at the single-cell level, to differentiate in vitro into multiple mesodermal, endodermal, and ectodermal lineages [5]. Moreover, when injected into the mouse blastocyst, they give rise to viable chimeric offspring, thus demonstrating their ability to incorporate into virtually all tissues in vivo [6]. Recently, adipose tissue has been identified as another source of multipotent adult stem cells [7–9]. From a cell therapy perspective, adipose tissue presents several advantages compared with bone marrow as it is very large and can be easily removed by surgery with little trouble. Several research teams have successfully isolated, from adipose tissue, cell populations that are able to differentiate into mesenchymal cell types. They have been termed adipose-derived adult stem [9], processed lipoaspirate [7], and adipose tissue-derived stromal cells [10]. Recently, Rodriguez et al. characterized human multipotent adipose-derived stem (hMADS) cells from the stroma-vascular fraction isolated from infant adipose tissue [8, 11, 12]. After being cultured for more than 100 population doublings, these cells display a normal diploid karyotype. They retain multilineage differentiation potential as they undergo differentiation into adipocytes, osteoblasts, and myocytes in vitro [8, 11, 12]. Moreover, after transplantation into skeletal muscle of dystrophin-

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deficient (*mdx*) mice, they are able to restore long-term expression of dystrophin [13]. Thus, hMADS cells provide a powerful system for studying commitment and differentiation toward various lineages, as well as for cell therapy.

In addition to plasticity, self-renewal is a crucial feature of stem cells. It is defined by the ability to proliferate while maintaining an undifferentiated phenotype. The importance of self-renewal is best illustrated in vivo for hematopoietic stem cells (HSCs), which are able to give rise to both HSC and differentiated cells. This enables HSCs to perpetuate themselves while retaining the ability to regenerate mature blood cells throughout the entire lifespan of an organism [14]. In vitro, self-renewal has been defined by the ability of a cell to be maintained for many passages with no alterations of its developmental potential. It is still uncertain, however, how self-renewal is maintained in vivo and in vitro. The first clues have come from mouse embryonic stem (ES) cells, which appear to require extracellular stimuli such as leukemia inhibitory factor and bone morphogenic protein 4 to self-renew [15–17]. A few intrinsic mediators of pluripotency have been identified in these cells, including Oct4, Sox2, and Nanog [16–18]. Whether the mechanisms involved in self-renewal in embryonic versus adult stem cells are conserved is not known. A few signaling pathways have been shown to be involved in the regulation of adult stem cell self-renewal. Whereas the Notch and Wnt pathways participate in the self-renewal of hematopoietic, intestinal epithelial, and neural stem cells [19, 20], Hedgehog may play a role in the maintenance of neural stem cells in several regions of the mammalian central nervous system [21, 22]. Previous reports have identified the Wnt pathway as a promoter of proliferation and inhibitor of osteogenesis in mesenchymal stem cells [23–26]. However, no clear data demonstrate its role in self-renewal of these cells. Thus, the molecular mechanisms responsible for self-renewal of mesenchymal stem cells remain unclear at present. As in vitro expansion of mesenchymal stem cells is necessary for subsequent engraftment, it is crucial to identify intrinsic and extrinsic factors that contribute to their propagation.

Among the large number of intrinsic and extrinsic factors that could affect self-renewal of mesenchymal stem cells, we focused our attention on the fibroblast growth factor (FGF) pathway. This pathway, and especially FGF2, has been identified as a major candidate for self-renewal regulation in human ES cells [27–29]. FGF2 may also be important for maintaining the neural stem cells pool in the mouse brain subventricular zone [30]. Moreover, FGF2 increases lifespan of bone marrow stromal cell primary cultures when cultivated at low cell density [31] and has been reported to support proliferation as well as the osteogenic and chondrogenic differentiation potential of these cells [32–35]. Finally, a transcriptome screening of undifferentiated versus differentiated hMADS cells revealed that this factor is expressed by undifferentiated cells but not by their differentiated derivatives, which suggests that it might be involved in self-renewal (unpublished data). In addition to these proliferative properties, FGF2 was shown to mediate osteoblast, chondrocyte, and neuron differentiation [36–38], as well as endothelial cell migration [39]. Thus, its role seems to be context-dependent, and additional investigations are required to clearly identify the role of FGF2 in mesenchymal stem cells.

FGF2 belongs to the 22-member family of fibroblast growth factors. Five isoforms of FGF2 have been characterized, representing alternative translation products from a single mRNA. The 22-, 22.5-, 24-, and 34-kDa isoforms have been found to localize in the nucleus and trigger an active intracrine signaling pathway, whereas the AUG-initiated isoform of 18-kDa is mostly cytosolic [40, 41]. FGF2 binds to two classes of receptors: high-affinity transmembrane receptor tyrosine kinases (fibroblast growth factor receptors [FGFRs]) and low-affinity receptors, which are heparan sulfate proteoglycans (HSPGs). The *FGFRs* belong to a family of five genes (*FGFR1–FGFR5*), from which alternative splicings generate several isoforms. HSPGs not only are involved in FGF2 storage and protection from proteolysis but also provide a higher affinity of FGF2 for FGFRs [42, 43].

In this study, we investigated the role of FGF2 in hMADS cell self-renewal capacity in vitro. We assessed self-renewal by measuring the proliferation rate, the ability to grow at the single-cell level, and the differentiation potential of hMADS cells. The data presented here clearly identify FGF2 as a key factor in the maintenance of hMADS cell self-renewal and highlight some of the transduction pathways involved in this process.

Materials and Methods

Cell Culture

hMADS cells were obtained from the stroma of human adipose tissue as described previously [13]. The cell populations that have been extensively studied were isolated from the pubic region fat pad of a 5-year-old male donor (hMADS2) and a 4-month-old male donor (hMADS3) [13]. Proliferation medium was composed of Dulbecco's modified Eagle's medium (low glucose) containing 10% fetal calf serum (FCS), and 100 U/ml penicillin and streptomycin. After reaching 90% confluence, adherent cells were dissociated in 0.25% trypsin EDTA and seeded at 4,500 cells per cm^2. PD173074 was used to inhibit FGFR signaling. Final concentration (75 nM) was determined as being the lowest concentration that inhibits the proliferative effect of 2 ng/ml of FGF2. At that concentration, no toxicity and no effect on epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) pathways were observed (data not shown). Cultures were maintained at 37°C in a humidified gassed incubator, 5% CO_2 in air. Media were changed every other day.

hMADS Cell Differentiation

Adipocyte differentiation were performed as described previously [13]. Cells were plated at high density (15,000 cells per cm^2), and differentiation was induced 24 hours after plating. Glycerol-3-phosphate dehydrogenase (GPDH) activity was performed in triplicate wells, using the method described previously [44]. GPDH is an enzyme that is required for the formation of triglycerides. Oil red O staining was performed as described previously [45].

Cell Proliferation Assays

hMADS cells were plated into 100-mm diameter dishes (2.5×10^5 cells per dish). After the appropriate time, cells were

trypsinized as described above and counted with a Coulter counter.

Clonal Assays

Cells were plated at a density of 10 cells per cm^2 in 100-mm^2 dishes. Fifteen days after plating, cells were fixed with 0.25% glutaraldehyde and stained with 0.1% crystal violet. Colonies containing at least 40 cells were counted under a light microscope. Medium was changed three times a week.

Reverse Transcription-Polymerase Chain Reaction Analysis

Total RNA was extracted using TRI-Reagent kit (Euromedex, Souffelweyersheim, France, http://www.euromedex.com) according to the manufacturer's instructions, and reverse transcription-polymerase chain reaction (RT-PCR) analysis was conducted as described previously [11]. All primers sequences are detailed in supplemental online Table 1. An aliquot of PCR products was analyzed on 2% ethidium bromide-stained agarose. For quantitative PCR, final reaction volume was 25 μl, including specific primers (0.4 μM), 5 ng of reverse-transcribed RNA, and 12.5 μl of SYBR green master mix (Eurogentec, Angers, France, http://www.eurogentec.com). Quantitative PCR conditions were as follows: 2 minutes at 50°C; 10 minutes at 95°C; and 35 cycles of 15 seconds at 95°C, 1 minute at 60°C. Real-time PCR assays were run on an ABI Prism 7700 real-time PCR machine (PerkinElmer Life and Analytical Sciences, Boston, http://www.perkinelmer.com).

Enzyme-Linked Solid Phase Immunosorbent Assay

Quantikine enzyme-linked solid phase immunosorbent assay (ELISA) kit (R&D Systems Inc., Minneapolis, http://www.rndsystems.com) was used to detect FGF2 in cell culture supernatants, fetal calf serum, and cell surface washes. Cells were washed with 2 M NaCl (20 mM HEPES, pH 7.4) for 5 seconds to remove HSPG-bound FGF2.

Preparation of Cell Extracts and Western Blot Analysis

Whole cell extracts, SDS-polyacrylamide gel electrophoresis, blotting, and enhanced chemiluminescence were performed as described previously [45]. Primary antibodies were goat anti-FGF2 (R&D Systems), mouse anti-phospho-ERK1/2, and rabbit anti-ERK1/2 (Cell Signaling Technology, Beverly, MA, http://www.cellsignal.com). Secondary horseradish peroxidase-conjugated antibody was purchased from Promega (Madison, WI, http://www.promega.com).

Materials and Chemicals

Cell culture media and reagents were purchased from Gibco-BRL (Gaithersburg, MD, http://www.gibcobrl.com), and FCS was purchased from Dutscher S.A. (Brumath, France, http://www.dutscher.com). FGF2 was from Peprotech (Rocky Hill, NJ, http://www.peprotech.com). Rosiglitazone (BRL4953) was a gift from Dr J.F. Dole (GlaxoSmithKline, King of Prussia, PA, http://www.gsk.com). PD173074 was a gift from GlaxoSmithKline Research and Development Ltd. (Hertfordshire, U.K.). U0126 was purchased from Sigma-Aldrich (St. Louis, http://www.sigmaaldrich.com).

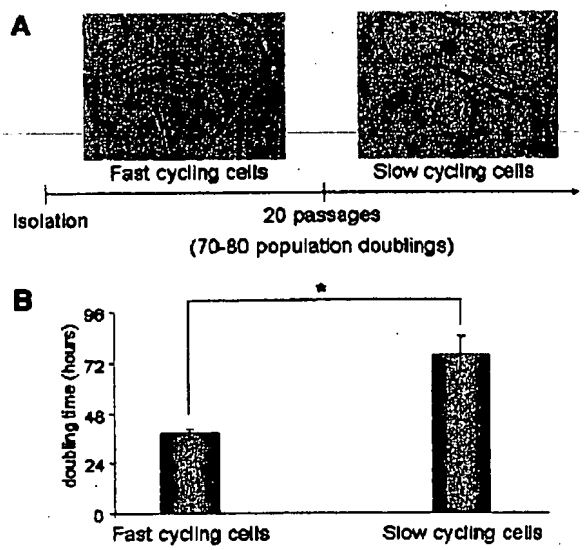

Figure 1. Overview of the change in proliferation status and morphology of hMADS cells as the number of population doublings increases. (A): Light microscopy photographs of fast- and slow-cycling human multipotent adipose-derived stem cells stained with crystal violet. Scale bars = 75 μm. (B): Average population doubling time of fast- and slow-cycling cells. *, $p < .05$.

Results

hMADS Cells Are Dependent on FGF2 Supplementation for Long-Term Propagation in Culture

Four populations of hMADS cells were isolated from different donors (hMADS1, hMADS2, hMADS3, and hMADS6). Two of them (hMADS2 and hMADS3) were extensively studied and yielded similar results. They were shown to be able to proliferate in culture for more than 30 passages, which represents approximately 150 population doublings, without reaching senescence [13]. However, during propagation in vitro, we noticed that the cell morphology changed dramatically, from spindle-shaped cells to large and flat cells (Fig. 1A). This morphological change was accompanied by a change in cell proliferation ability. Two major stages of cell proliferation could be identified: a "fast-cycling" stage, which extended until population doubling 70–80, followed by a "slow-cycling" stage. Fast-cycling cells exhibited an average population doubling time of 36 hours, whereas slow-cycling cells only divided every 72–96 hours (Fig. 1B), despite the presence of 10% FCS in culture medium. Thus, although FCS contains a broad range of growth factors, it was not sufficient to maintain hMADS cells in a fast-cycling state.

In an effort to find culture conditions to maintain hMADS cells in a fast-cycling state, growth factors such as FGF2, EGF, and PDGF were added separately to serum-containing medium. FGF2 only was able to sustain proliferation over several passages (data not shown). It is noteworthy that FCS did not contain FGF2, as checked by ELISA (data not shown). As shown in Figure 2A, FGF2 was able to maintain hMADS cells in a fast-cycling state until at least passage 25. During this time

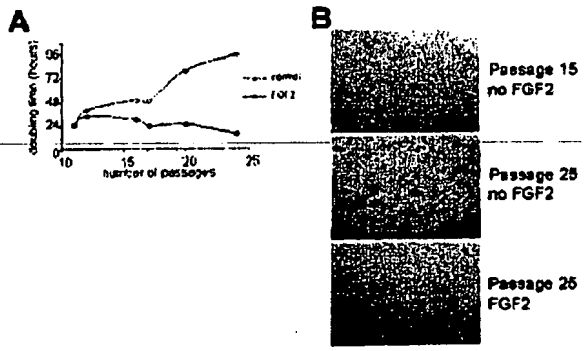

Figure 2. Effect of FGF2 on proliferation and morphology of human multipotent adipose-derived stem (hMADS) cells during in vitro propagation. (A): Comparative evolution of doubling times of hMADS cells cultured with or without 2 ng/ml FGF2. (B): Light microscopy photographs of hMADS cell maintained with or without 2 ng/ml FGF2. Scale bars = 350 μm. Abbreviation: FGF2, fibroblast growth factor 2.

course, hMADS cells retained their initial spindle-shaped morphology (Fig. 2B).

FGF2 Allows hMADS Cells to Retain Their Differentiation Potential During In Vitro Expansion

The maintenance of a differentiation potential during propagation is a critical component of self-renewal. To assess hMADS cell differentiation potential during in vitro propagation, we focused on their ability to differentiate into their three major derivatives (e.g., adipocytes, osteoblasts, and chondrocytes).

As illustrated in Figure 3A, the ability of hMADS cells from two different donors to differentiate into adipocytes was decreased in slow-cycling cells compared with fast-cycling cells. However, treatment of slow-cycling cells with FGF2, exclusively during propagation, completely restored their adipogenic potential, as monitored by GPDH activity (Fig. 3B; supplemental online Fig. 1).

Similar results were obtained when hMADS cells were induced to differentiate into osteoblasts and chondrocytes (supplemental online Fig. 2): differentiation potential decreased in slow-cycling cells but could be maintained when these cells were cultivated with FGF2. Together, these data indicate that FGF2 prevents the loss of differentiation capacity observed during hMADS cell long-term propagation.

FGF2 Allows hMADS Cells to Maintain Their Ability to Propagate at the Single-Cell Level

The ability to expand at the single-cell level in vitro is a major feature of self-renewing cells. The clonogenic potential of hMADS cells was assessed at different propagation time points. Fast-cycling cells were able to form clones with an efficiency of (11.6% ± 0.08%), whereas slow-cycling cells exhibited lower clonogenic efficiency (5.7% ± 0.04%). Addition of FGF2 to slow-cycling cells almost completely restored their clonogenic potential, raising the clonogenic efficiency to 10.1% ± 0.27 (Fig. 4). Therefore, FGF2 improves the ability of long-term cultured cells to proliferate at the single-cell level.

Together, these data indicate that fast-cycling hMADS cells display the main characteristics of self-renewing multipotent stem cells. They lose this property and turn into slow-cycling

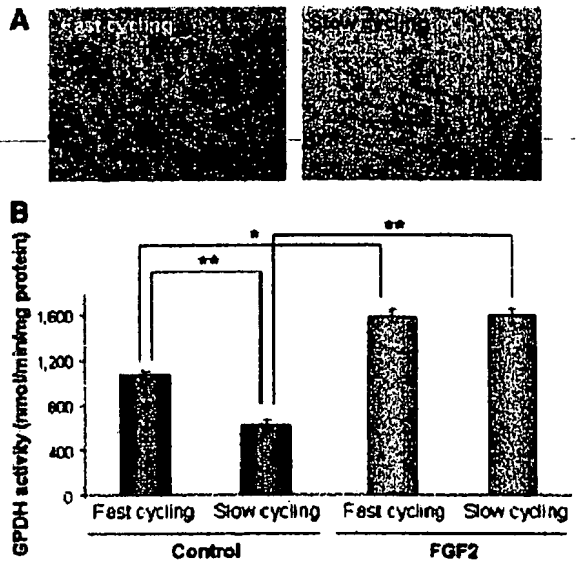

Figure 3. Effect of FGF2 on differentiation potential of fast- and slow-cycling human multipotent adipose-derived stem (hMADS) cells. Fast- and slow-cycling cells were maintained 5 days with or without (control) 2 ng/ml FGF2 during propagation. Then, FGF2 was removed, and 36 hours later, cells were induced to undergo differentiation into adipocytes. (A): Light microscopy photographs of fast- (passage 15) and slow-cycling (passage 25) hMADS cells that were maintained in the absence of FGF2 and then subjected to adipogenesis for 10 days. Lipid droplets accumulation was revealed by oil red O staining. Scale bars = 350 μm. (B): Adipogenesis, quantified by GPDH activity measurement, of fast- and slow-cycling cells pretreated with FGF2 or not pretreated. Columns indicate mean ± SE (n = 3). *, $p < .05$; **, $p < .01$. Abbreviations: FGF2, fibroblast growth factor 2; GPDH, glycerol-3-phosphate dehydrogenase.

cells during propagation in culture. Nevertheless, FGF2 supplementation prevents this process, allowing hMADS cells to be propagated at a multipotent state.

Acquisition of Slow-Cycling State Involves Irreversible Decrease in FGF2 Expression

To explain why slow-cycling cells required exogenous FGF2 to self-renew whereas fast-cycling cells did not, we hypothesized that the latter may initially express and secrete FGF2, whereas slow-cycling cells may have lost this ability. We therefore investigated the expression of FGF2 protein in both types. As illustrated in Figure 5A, slow-cycling cells displayed a marked decrease in the expression of FGF2 (isoforms of 18, 22/22.5, and 24 kDa) compared with fast-cycling cells. It is worth mentioning that FGF2 treatment of hMADS cells allowed the maintenance of their self-renewal abilities but did not sustain their FGF2 expression (Fig. 5A). This suggests that unlike the decrease in proliferation and differentiation, the decrease in FGF2 expression became irreversible in slow-cycling cells.

We next checked whether fast-cycling cells express FGFRs by RT-PCR. Only one receptor, FGFR1, was detected (Fig. 5B). Quantitative PCR analysis showed that this receptor was equally expressed in fast- and slow-cycling cells (supplemental online Fig. 2).

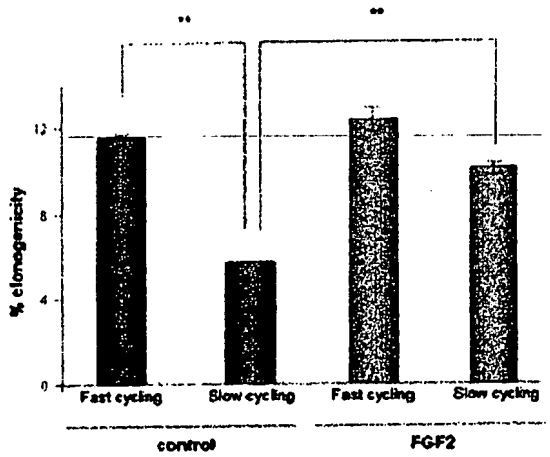

Figure 4. Effect of FGF2 on human multipotent adipose-derived stem (hMADS) cell clonogenicity. Fast- (passage 15) and slow-cycling (passage 25) cells were seeded at low density (10 cells per cm^2) and maintained in proliferation medium containing or not containing FGF2 (2 ng/ml). After 15 days, colonies containing more than 40 cells were scored. Results are expressed as the ratio of the number of scored colonies over the initial number of seeded cells. Similar clonogenicity measurements were performed on hMADS cells from three other donors and yielded similar results. Columns indicate mean ± SE ($n = 3$). **, $p < .01$. Abbreviation: FGF2, fibroblast growth factor 2.

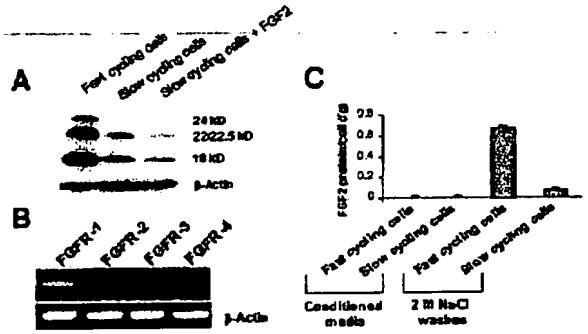

Figure 5. Expression of FGF2 and FGFR1 by human multipotent adipose-derived stem (hMADS) cells. (A): Western blot for FGF2. Fifty micrograms of hMADS cells protein extracts of fast- (passage 15) and slow-cycling (passage 25) cells were resolved on a 15% polyacrylamide gel, transferred, and blotted with anti-FGF2 antibody. The right lane corresponds to cells treated with FGF2 from passages 15–25. (B): Screening for FGFR expression was performed by conventional reverse transcription-polymerase chain reaction. (C): Secretion of FGF2 by fast- and slow-cycling hMADS cells was assessed by enzyme-linked immunosorbent assay. One hundred microliters of 48-hour conditioned media and 100 μl of 2 M NaCl 20 mM HEPES (pH 7.4) washes were assayed according to the manufacturer's instructions. Columns indicate mean ± SE ($n = 3$). Abbreviations: FGF2, fibroblast growth factor 2; FGFR, fibroblast growth factor receptor; kD, kilodaltons.

The expression of FGF2 and FGFR1 by hMADS cells suggests the existence of an active autocrine/paracrine loop. Therefore, we checked for the secretion of FGF2 by hMADS cells by performing an ELISA on conditioned media from fast- and slow-cycling cells. As shown in Figure 5C, no detectable

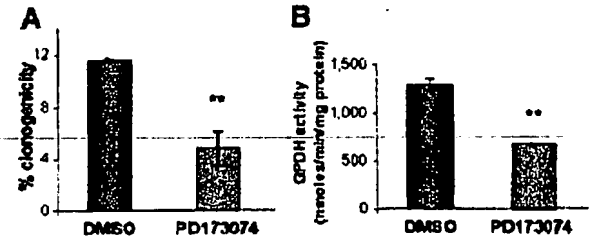

Figure 6. Effect of PD173074 on clonogenicity and differentiation potential of fast-cycling (passage 15) human multipotent adipose-derived stem (hMADS) cells. (A): Effect of PD173074 on the clonogenicity of fast-cycling cells. Cells were treated with PD173074 (75 nM) or not treated during the clonal assay and then analyzed as described in Figure 4. (B): Effect of PD173074 treatment during proliferation on adipogenesis of fast-cycling hMADS cells. Cells were seeded and treated with PD173074 (75 nM) during 5 days and then processed as described in Figure 3. Columns indicate mean ± SE ($n = 3$). **, $p < .01$. Abbreviations: DMSO, dimethyl sulfoxide; GPDH, glycerol-3-phosphate dehydrogenase.

levels of FGF2 were found in any conditioned medium. This could reflect an association of FGF2 to cell surface, presumably to HSPG, rather than a failure to export FGF2 through the plasma membrane [46]. To remove putative interactions between HSPGs and FGF2, cell layers were washed with 2 M NaCl at neutral pH. Significant amounts of FGF2 were detected in washes from fast-cycling cells, whereas only very low amounts of FGF2 were found in those from slow-cycling cells (Fig. 5C). Together, these results show that hMADS cells express a FGF2 autocrine signaling loop that irreversibly declines as the cells are expanding. This decline results, at least in part, from a decrease in FGF2 production, which may explain why slow-cycling cells become dependent on FGF2 supplementation to maintain their self-renewal ability.

FGF2 Autocrine Signaling Is Necessary for hMADS Cell Self-Renewal During In Vitro Propagation

To demonstrate that the autocrine FGF2 signaling was necessary for their self-renewal, hMADS cells were cultured in the presence of PD173074, a specific inhibitor of FGF receptor phosphorylation [47], and then were assessed for their clonogenic and differentiation potential.

When seeded at clonal density in the presence of PD173074, fast-cycling cells exhibited a potent decrease in their clonogenic efficiency compared with nontreated cells (Fig. 6A). Thus, signaling through FGFR1 promotes hMADS cell expansion at the single-cell level. When fast-cycling cells were treated for one passage with PD173074 and then induced to differentiate, adipogenesis was strongly impaired compared with untreated cells (Fig. 6B). In conclusion, the inhibition of FGF2 signaling in fast-cycling cells impairs both their clonogenic potential and their differentiation abilities, indicating its crucial role in the regulation of hMADS cell self-renewal.

The ERK1/2 Pathway Is Partly Responsible for FGF2-Induced Self-Renewal

We next tried to identify signaling pathways that could mediate FGF2 effects on hMADS cell self-renewal. Because in many

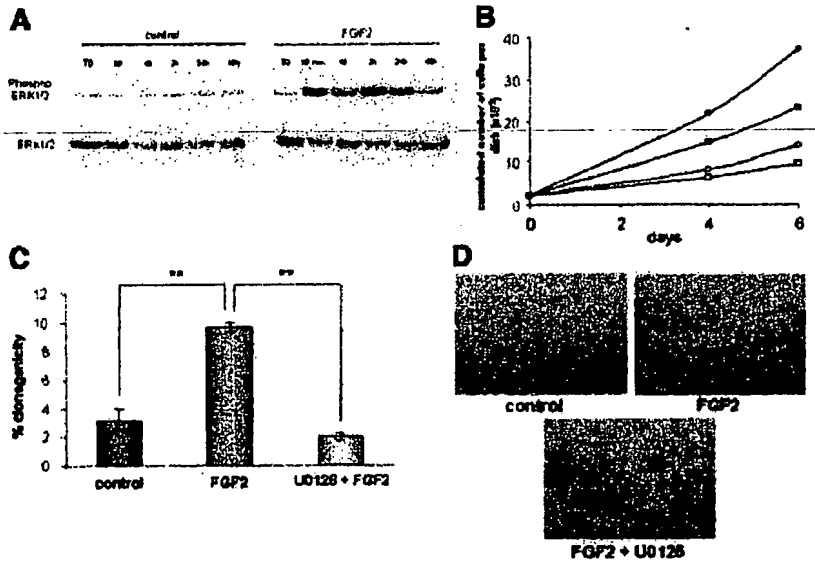

Figure 7. Involvement of the ERK1/2 pathway in FGF2-mediated self-renewal. (A): Western blot against phospho-ERK1/2 and ERK1/2 on FGF2 stimulated slow-cycling human multipotent adipose-derived stem (hMADS) cell (passage 25) extracts. Thirty micrograms of total extracts were resolved on a 12% polyacrylamide gel and then blotted with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. (B): Cumulative number of slow-cycling hMADS cells (passage 25) propagated for three passages under the following conditions: control (open circles), 2 ng/ml FGF2 (filled circles), 10 μM U0126 (open squares), or 2 ng/ml FGF2 and 10 μM U0126 (filled squares). (C): Clonogenicity efficiency of slow-cycling hMADS cells (passage 25) treated either with 2 ng/ml FGF2 or simultaneously with 10 μM U0126 and 2 ng/ml FGF2. Columns indicate mean ± SE ($n = 3$). **, $p < .01$. (D): Light microscopy photographs of slow-cycling hMADS cells (passage 25) that were treated either with 2 ng/ml FGF2 or simultaneously with 10 μM U0126 and FGF2 during propagation (5 days) and then processed as described in Figure 3. Lipid droplet accumulation was revealed by oil red O staining. Scale bars = 350 μm. Abbreviations: ERK, extracellular signal-related kinase; FGF2, fibroblast growth factor 2.

cell types the ERK1/2 pathway is known to be activated by FGF2, we investigated its role in hMADS cell self-renewal.

Addition of FGF2 to slow-cycling hMADS cells induced ERK1 and ERK2 phosphorylation. This activation was first observed after a 10-minute exposure, and the levels of phosphorylated ERK1/2 started to decrease after 24 hours (Fig. 7A).

To assess whether the activation of the ERK1/2 pathway was necessary for the effect of FGF2 on hMADS cell self-renewal, we evaluated the effect of U0126 on FGF2-mediated proliferation, clonogenicity, and differentiation potential. U0126 specifically inhibits mitogen-activated protein kinase kinase 1, which is the mitogen-activated protein kinase kinase that activates ERK1/2.

As illustrated in Figure 7B, treatment of slow-cycling hMADS cells with U0126 alone did not significantly alter their proliferation rate in the absence of FGF2. In contrast, FGF2-mediated increase in proliferation was counteracted in the presence of U0126. Furthermore, the clonogenic potential of slow-cycling hMADS cells was also impaired by simultaneous treatment with FGF2 and U0126 (Fig. 7C). Thus, these results suggest that FGF2 promotes proliferation and clonogenicity of hMADS cells, at least in part, by activating the ERK1/2 pathway.

Finally, we evaluated the effect of U0126 on the maintenance of adipocyte differentiation potential mediated by FGF2. Slow-cycling hMADS cells were treated with FGF2 in the absence or presence of U0126 for two passages and then were induced to differentiate. The results shown in Figure 7D indicate that U0126 treatment during hMADS cell proliferation did not affect FGF2-mediated maintenance of differentiation potential. These results were confirmed by GPDH activity assay (data not shown). In conclusion, these data suggest that the ERK1/2 pathway is partly responsible for FGF2-mediated self-renewal of hMADS cells, as it is involved in proliferation and clonogenic efficiency but not in the maintenance of differentiation potential.

Discussion

Given their convenient isolation and extensive proliferative capacities in vitro, adipose tissue-derived stem cells are a promising source of human stem cells for regenerative medicine. Nevertheless, little characterization of their self-renewal properties has been achieved so far. Indeed, to generate sufficient amounts of stem cells for transplantation, one should be able to expand them in vitro without altering their developmental potential. This is a major difficulty with human bone marrow stem cell expansion. These cells are plastic at early passages but lose their multipotency as they are propagated in culture. For instance, they seem to lose their adipogenic potential upon reaching passage 12 in culture [48]. We also observe a progressive loss of differentiation potential during expansion of adipose tissue-derived stem cells in serum-containing medium. We show herein that a decrease in FGF2 signaling is responsible not only for this decline in differentiation capacity but also for a reduced ability of the cells to grow at the single-cell level. Exogenous FGF2 restored both of these properties, indicating that FGF2 plays a crucial role in self-renewal of adipose tissue-derived stem cells during propagation in vitro.

To our knowledge, there has been only one recent report dealing with the expansion of bone marrow mesenchymal stem cells in fully chemically defined conditions [49], which would be best suited for subsequent therapeutic use of the cells. Still, expansion of adipose tissue-derived mesenchymal stem cells under these kinds of conditions has never been described. The media that are currently used contain serum at high concentra-

tions, which includes a broad range of growth factors. Our data presented here indicate that the widely used 10% FCS-containing media may not be optimal for long-term propagation of mesenchymal stem cells. Indeed, adipose tissue-derived stem cells cultured under these conditions progressively lose FGF2 expression, resulting in an impairment of their self-renewal ability. Whether long-term exposure to high serum concentration itself is responsible for the decrease in FGF2 expression is unclear at present. Nevertheless, exposure to high serum concentrations has previously been reported to impair propagation of primary rodent cells, including Schwann cells and oligodendrocyte precursor cells [50, 51]. In these cell types, impairment of propagation was attributed to telomere-independent premature senescence. In contrast, the progressive loss of self-renewal ability of adipose tissue-derived stem cells during propagation does not seem to be caused by senescence, since the cells display no senescence-associated β-galactosidase activity [13] and since this process is fully reversible upon addition of FGF2. However, even though hMADS cells are able to retain their stem cells features during extensive propagation in medium containing high serum concentrations in the presence of FGF2, it would be worth optimizing fully chemically defined conditions allowing efficient propagation of undifferentiated mesenchymal stem cells. Defined conditions would allow convenient identification of factors involved in self-renewal and would probably cause fewer damages to the cells than high serum exposure during long-term propagation. Preliminary data suggest that hMADS cells can be propagated in low-serum conditions and that FGF2 is necessary for maintenance of their self-renewal abilities under these conditions (data not shown).

Self-renewal implies the coordination of two features: proliferation and maintenance of differentiation capacities. These two processes are not necessarily regulated by a common pathway. In the hematopoietic system, for instance, HOXB4 appears to regulate proliferation of HSCs, whereas the Notch pathway regulates their differentiation potential [52]. Our data concerning the ERK1/2 pathway suggest that FGF2 controls proliferation and differentiation of adipose tissue-derived stem cells through distinct downstream effectors. Indeed, we showed that the ERK1/2 pathway is involved in FGF2-mediated clonogenicity of hMADS cells but not in the maintenance of their differentiation potential. Moreover, the observation that exogenous FGF2 does not improve clonogenicity of fast-cycling cells, whereas it increases their differentiation potential (Figs. 3B, 4), corroborates the hypothesis that distinct transduction pathways act to promote clonogenicity versus maintenance of the differentiation potential. The respective pathways and targets genes responsible for these two features remain to be elucidated.

In conclusion, our study identifies for the first time FGF2 as a key factor for long-term propagation and self-renewal of human adipose tissue-derived stem cells. If the understanding of the self-renewal of adipose tissue-derived stem cells in vitro is crucial for therapeutic use of these cells, one should also consider the importance of this process in vivo. As these cells are isolated from adipose tissue and are able to differentiate into adipocytes, they might constitute a reservoir for adipocyte precursors. This pool of precursor cells may be responsible for fat mass enlargement according to their ability to proliferate and then to differentiate after stimulation by the appropriate signals [53]. It has been reported that FGF2 is expressed in human adipose tissue [54]. Since this factor is able to enhance proliferation and to maintain adipogenic potential of hMADS cells, it could play an important role in physiological and pathological modifications of adipose tissue, such as in aging and obesity.

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DISCLOSURES

The authors indicate no potential conflicts of interest.

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Chondrogenic differentiation of bovine bone marrow mesenchymal stem cells in pellet cultural system

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Objective. **Pluripotent mesenchymal stem cells (MSC) have been isolated and well characterized from several tissue sources, including bone marrow stroma. MSC from different animals showed slight differences in morphology and in the potential to differentiate. In the present study, we isolated MSC from bovine bone marrow and induced chondrogenesis in order to establish a new experimental model of stem cell research.**

Methods. **Bone marrow was harvested from 8 calves. For inducing chondrogenesis, MSC were cultured in pellet culture system in a chemically defined medium supplemented with 0 and 10 ng/mL of transforming growth factor β1 (TGF-β1). Chondrogenic differentiation was evaluated by histological, immunohistochemical, and in situ hybridization techniques. The degrees of genes expression were measured by quantitative RT-PCR.**

Results. **Metachromatic alcian blue staining and immunoreactivity for type II collagen were detected in both pellet groups (0 and 10 ng/mL TGF-β1) after 7 days of culturing. In situ hybridization demonstrated strong expression of type II collagen and aggrecan mRNAs in the round cells located at the center region of pellets and at densely organized areas. On the other hand, type I collagen mRNA was strongly expressed in the superficial layer of the pellets. After 20 days of pellet culture, expression of type II collagen mRNA in the cells which were not treated by TGF-β1 was 1.7-fold higher compared with that treated by TGF-β1.**

Conclusion. **Independent, spontaneous chondrogenesis of bovine MSC in pellet culture occurred without addition of any external bioactive stimulators, namely factors from TGF-β family, which were previously considered necessary.** © **2004 International Society for Experimental Hematology. Published by Elsevier Inc.**

Adult stem cells have been isolated and well characterized from several tissue sources including bone marrow [1], central nervous system [2,3], retina [4], skeletal muscles [5], dermis of mammalian skin [6], and adipose tissue [7]. In the past three decades, most of the work was focused on mesenchymal stem cells (MSC) found within the bone marrow stroma. Bone marrow MSC were purified and expanded and multilinage differentiation was induced in a number of species including rat [8], rabbit [9], dog [10], human [1,11], horse [12], pig [13], and cat [14]. For inducing differentiation, MSC should be cultivated under appropriate culture conditions and stimulated with some bioactive factors. Many growth factors, including transforming growth factor β1 (TGF-β1), bone morphogenic protein (BMP), fibroblast growth factor (FGF), and insulin-like growth factor (IGF-I), have been evaluated for the potential to enhance chondrogenesis [15].

Defects of the cartilage have been known to be poor healing injuries, which treated by conventional surgical methods do not lead to hyaline cartilage regeneration. For this purpose, transplantation of allogeneic or autologous pluripotentional or mature-differentiated cells that are expanded ex vivo have been attempted to cure such defects.

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Transplantation of autologous chondrocytes is limited by lack of suitable donor sites from where large cartilage samples could be harvested, and by limited potential of mature chondrocytes to be expanded in vitro without losing their phenotype [16]. These disadvantages can be substituted by application of the bone marrow MSC in repairing articular cartilage defects. Experimental results, in which rabbit model was used, have been shown that autologous bone marrow MSC transplanted into artificial osteochondral defects were able to differentiate into chondrocytes and repair the defects with hyaline cartilage [9].

Bone marrow MSC harvested from different species displayed slight differences in morphology and differentiation potential. The number of MSC found in canine bone marrow is fourfold higher than in humans. Canine MSC have a much faster doubling time, but lose the osteogenic potential after second passage [10]. Furthermore, canine MCS did not form calcificated nodules during osteogenesis in vitro, which was observed in human and rat MSC [10]. During osteogenesis induced by dexamethasone, feline bone marrow MSC did not express alkaline phosphatase (ALP) [14], or human MSC did not express osteocalcin mRNA [17]. Dexamethasone is a potent inducer of osteocalcin and ALP, which have been reported to be typical markers of osteogenic differentiation in other species [10,13,16]. MSC harvested from commonly used strains of inbred mice displayed variations in yield, growth, and differentiation [18]. Considering those and other findings, it could be concluded that differentiation processes and mechanisms that are valid for MSC from one species are not necessarily valid for others. Besides species character, bone marrow donor, isolation method, in vitro cell expansion technique, media formulation, and other factors also must be considered in experiments with MSC [19–21].

The majority of research concerning MSC has been done on rodents or humans. Small experimental animals can be used to research the principles of stem cell transplantation therapy, but prior to transferring this technology to the therapy it is important to introduce it to a large-animal model, which is biomechanically more relevant to humans [10,22–24]. Cattle are considered large-animal experimental models, and as such give numerous advantages for making progress in clinical application of MSC to human medicine, especially in musculoskeletal health problems. The bovine animal model, which we introduced for the first time in the stem cell research, is a potential donor of relatively large amounts of bone marrow aspirate, and consequently leads to sufficient yield of isolated MSC, providing further opportunity to use it in numerous in vitro and in vivo experiments.

The objective of this study is to evaluate suitable methods for bovine MSC isolation, in vitro expansion, and chondrogenic differentiation, and to facilitate further in vitro studies and in vivo experiments for resurfacing cartilage defects using the bovine model.

Materials and methods

Materials

All reagents and supplements were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise noted.

Harvest and isolation of bovine MSC and chondrocytes

Bone marrow was obtained from tuber coxae, femur, crista tibia, and proximal end of the humerus of 8 Holstein Friesian calves, ranging from 2 days to 6 months old. The marrow was drawn into 25-mL syringes containing 1000 units of heparin following aseptical preparation of the harvesting fields and infiltration with local anesthetic.

Bovine MSC were isolated by modification of methods previously described [15,16,25,26]. Briefly, one volume of bone marrow sample was mixed with two volumes of phosphate-buffered saline (PBS), and the mixture was centrifuged at 300*g* for 10 minutes. The supernatant was discarded, and the pellet was washed two more times with Dulbecco's Modified Eagle Medium (DMEM; GIBCO BRL, Grand Island, NY, USA). After determination of the cell viability and the number using trypan blue staining, following lysis of erythrocytes by the addition of 4% acetic acid, 5×10^4/cm^2 nucleated cells were plated in T-75 culture flasks in DMEM (low glucose) containing: penicillin G 100 U/mL, streptomycin 100 μg/mL, amphotericin B 0.25 μg/mL, HEPES 2.4 mg/mL, $NaHCO_3$ 3.7 mg/mL, and 10% fetal bovine serum (FBS; lot No. 5300C, ICN Biomedicals, Aurora, OH, USA). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 4 days of culturing, the nonadherent cells were removed by changing the culture media. Following the initial 4 days, which were necessary for cells to attach to the flask, medium was changed every 2 to 3 days. On days 12 to 13, cells were detached (0.25% trypsin/0.1% EDTA) and replated according to the standard cultural technique at a 1:3 or 1:4 dilution (first passage). When cells began to reach near confluent stage, they were trypsinized and used for the preparation of pellets. Cell cultures from each calf were maintained separately.

Chondrocytes were isolated from the articular cartilage harvested from the same calf, from which bone marrow was aspirated. Briefly, cartilage pieces were digested with 0.1 mg/mL of collagenase (Wako Pure Chemical, Osaka, Japan) in DMEM/10% FBS for 18 hours at 37°C with gentle stirring followed by filtering through a 100-μm nylon mesh. Isolated primary chondrocytes were cultured in monolayer manner under same conditions as MSC for 20 days and were used for positive control [27,28].

Pellet culture

Cells from the first passage were resuspended in the serum-free chemically defined medium consisting of DMEM (high-glucose), insulin 6.25 μg/mL, transferin 6.25 μg/mL, selenious acid 6.25 μg/mL, bovine serum albumin 1.25 mg/mL, pyruvate 1 mM, linoleic acid 5.35 μg/mL, and ascorbate 2-phosphate 50 μg/mL. For preparation of each pellet, aliquots of 1×10^6 cells in 1 mL of defined medium were spun down at 500*g* for 10 minutes in a 15-mL polypropylene conical tube [29]. Pellets were divided into 2 experimental groups according to the presence of the TGF-β1 (0 and 10 ng/mL) (R&D Systems, Minneapolis, MN, USA) in the medium, and cultivated at 37°C in a humidified atmosphere including 5% CO_2 for 20 days by changing the medium every 2 days.

Histological and immunohistochemical analysis of the pellet

Pellets were harvested after 7, 10, and 20 days of culturing, fixed in 10% buffered formalin for 2 hours, embedded in 2% agarose for easy manipulation, and kept in 70% ethanol overnight. Samples were embedded in paraffin and 5-μm sections were cut. Hematoxylin and Eosin (H&E) staining of paraffin sections was done for evaluation of cell morphology in pellets. Sulfated glicosaminoglicans (GAG) were visualized by staining with 0.5% alcian blue for 10 minutes. Collagen type I and II were detected using polyclonal antibodies (LSL Co., Tokyo, Japan) as previously described [27]. Briefly, after deparaffinization, sections were predigested with trypsin at 37°C for 30 minutes to facilitate antibody access, endogenous peroxidase was quenched by the treatment of 0.3% H_2O_2 in methanol at room temperature for 30 minutes, and nonspecific antibody binding was blocked by incubation of sections in a 10% normal goat serum at 37°C for 30 minutes. Rabbit anti-bovine collagen type I and II diluted 1:500 and 1:1000 respectively in 0.01 M PBS (pH: 7.4) were applied as a primary antibody at 4°C, overnight. Sections were then incubated with the secondary antibody, swine anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark), for 60 minutes followed by rabbit PAP kit (DAKO). Collagen type I and II were visualized by the reactions with 0.05% diaminobenzidine containing 0.01% H_2O_2.

In situ hybridization

For in situ hybridization, pellets were embedded in OCT compound and frozen in liquid nitrogen. Frozen sections (10 μm) were cut and mounted on silane-coated glass slides. Two kinds of nonoverlapping 45-mer antisense oligonucleotide probes were synthesized to detect every mRNA of interest. The following gene sequences were used: for collagen type I nucleotide residues 2408–2453 and 4108–4153 (Gen Bank AB008683); collagen type II nucleotide residues 278–323 and 777–822 (Gen Bank X02420); and aggrecan nucleotide residues 5540–5585 and 6750–6795 (Gen Bank U76615). In situ hybridization was performed as previously described [30]. Briefly, the oligonucleotides were labeled with ^{35}S-adenosine thiotriphosphate to a specific activity of 0.5×10^9 dpm/μg DNA, using terminal deoxynucleotidyl transferase. Sections were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) for 15 minutes and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 10 minutes. Hybridization was performed at 42°C overnight by the reaction with labeled oligonucleotide probes at a final radioactivity of 0.5×10^7 cpm/mL in 50% formamide, which contained 30 mM Tris-HCl (pH 7.4), 0.6 M NaCl, 1 mM EDTA, 100 mM dithiothreitol, 1× Denhardt's solution, 0.25% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and 200 μg/mL yeast tRNA. Control hybridization was performed in the presence of 20-fold excess amount of unlabeled antisense probe. Slides were washed in 2× SSC containing 0.1% sarkosyl for 30 minutes at room temperature, followed by washing 2 times in 0.1× SSC/0.1% sarkosyl for 40 minutes at 55°C. Slides were dipped into autoradiographic emulsion (NTB-2, Kodak, Rochester, NY, USA), exposed at 4°C for 4 weeks, and counterstained with hematoxylin.

Measurement of mRNA level by quantitative PCR method

Expressions of mRNA for genes of interest in the MSC cultured in pellet culture for 1 and 20 days, and in MSC and chondrocytes cultured in monolayer for 20 days, were measured by quantitative PCR method. Total RNA was isolated using Trizol (Invitrogen, Life Technologies, Carlsbad, CA, USA) following the manufacturer's instruction. After samples were treated by DNase to remove possible contamination of genomic DNA, first-strand complementary DNA (cDNA) was synthesized from 1 μg total RNA using M-MLV reverse transcriptase (Invitrogen) with oligo $(dT)_{20}$ used as a primer in a 20-μL reaction mixture. The amount of cDNA was measured by quantitative PCR method using Smart Cycler System (Cepheid, Sunnyvale, CA, USA). The PCR reaction was carried out in a 25-μL final volume, which contained PCR buffer, 3 mM $MgCl_2$, 0.3 mM dNTP mixture, 0.3 μM of each primer, and Taq polymerase (1.25 units/tube) (Takara Biomedicals, Otsu, Japan). The primer sequences specific for bovine glyceraldehyde-3-phosphate dehydrogenase (GAPDH), aggrecan, and collagen type I and II (Table 1) genes were obtained from Gen Bank and were synthesized at System Science (Sapporo, Japan). Thermal cycling was carried out at 95°C for 5 seconds, 60°C for 15 seconds, and 75°C for 15 seconds. The amount of PCR product was estimated by measurement of the intensity of fluorescence of SYBR Green I interacted into the PCR product. The mRNA expression level of target genes was normalized by dividing with the mRNA level of GAPDH. Quality of the PCR products was checked by melting curve analysis and electrophoresis of the PCR product. In addition, the identity of each PCR product was confirmed by sequence analysis (ABI-PRISM 310 Genetic Analyzer, PE Biosystems, Foster City, CA, USA).

Results

Cell culture

Spindle bipolar to polygonal fibroblastic cells attached to the flask were observed after 4 days of culturing, at the first changing of the medium (Fig. 1A). After 10 to 13 days, the cell culture reached confluence and, at the places where cells overlap, exhibited more cuboidal morphology. Cells in the first or further passages showed uniform monolayer (Fig. 1C) and needed approximately 1 week to reach confluence.

Table 1. Chondrogenic marker genes used in quantitative PCR (Q-PCR)

Gene	Primer nucleotide sequence	Product size (bp)	Accession number
GAPDH	Forward 5′ CCT TCA TTG ACC TTC ACT ACA TGG TCT A Reverse 5′ TGG AAG ATG GTG ATG GCC TTT CCA TTG	127	U85042
Collagen I	Forward 5′ TGC TGG CCA ACC ATG CCT CT Reverse 5′ CGA CAT CAT TGG ATC CTT GCA G	120	AB008683
Collagen II	Forward 5′ ATC CAT TGC AAA CCC AAA GG Reverse 5′ CCA GTT CAG GTC TCT TAG AG	147	X02420
Aggrecan	Forward 5′ CAC TGT TAC CGC CAC TTC CC Reverse 5′ GAC ATC GTT CCA CTC GCC CT	303	U76615

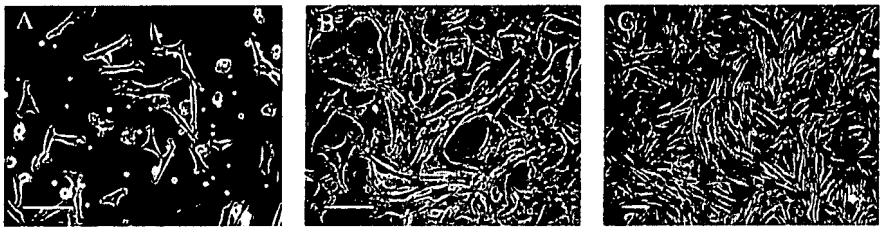

Figure 1. Morphology of bovine mesenchymal stem cells (MSC) cultured in monolayer manner on day 4 (**A**) and day 10 (**B**) in primary culture, and on day 7 (**C**) in first passage analyzed by phase-contrast microscope. Spindle bipolar to polygonal fibroblastic cells could be noticed after 4 days of culturing. Scale bar 100 μm.

In our experiment, cells from the first passage were used for pellet preparation. After centrifuging, a single compact pellet was formed within 1 to 2 days of incubation in a chemically defined medium. Approximately 85% of total prepared samples formed clearly identifiable pellets, which were further used in the experiment. The reason for unsuccessful pellet formation of some prepared samples might be unequal quality of isolated cells from different aspirations. Large differences of cell yield number and quality of MSC in aspirates from the same donor at the same time were previously reported, as some cell lines lose potential for differentiation with time, even under standard culture conditions [21,31].

Histology and immunohistochemistry

For histological and immunohistochemical evaluation, pellets were harvested after 7, 10, and 20 days of culturing. Pellets were divided into 2 experimental groups, according to the presence of TGF-β1 (0 and 10 ng/mL) in the culturing medium. On examination of paraffin sections stained with H&E, no notable differences in morphology of both pellet groups after the same period of culturing (Figs. 2 and 3) were detected. On day 10, the periphery of the pellet consisted of elongated cells arranged in a few layers, while the center region contained plump, rounded cells (Fig. 2E). On day 20, pellets retained the previous structure, except that much more extracellular matrix was observed surrounding the cells in the center of the pellets. Metachromatic staining with alcian blue, indicative for matrix of cartilage, was detected in all of the groups of pellets on day 7, 10, and 20. Staining was stronger in the center of the pellets on day 7, but this difference decreased over time of culturing. On day 7 in the center of the pellet, there were cells in lacunae with the appearance of hypertrophic chondrocytes surrounded by metachromatic stained matrix. This morphological modification of the cells was time dependent, and on day 20 all of the cells in the pellet had chondrogenic appearance, except

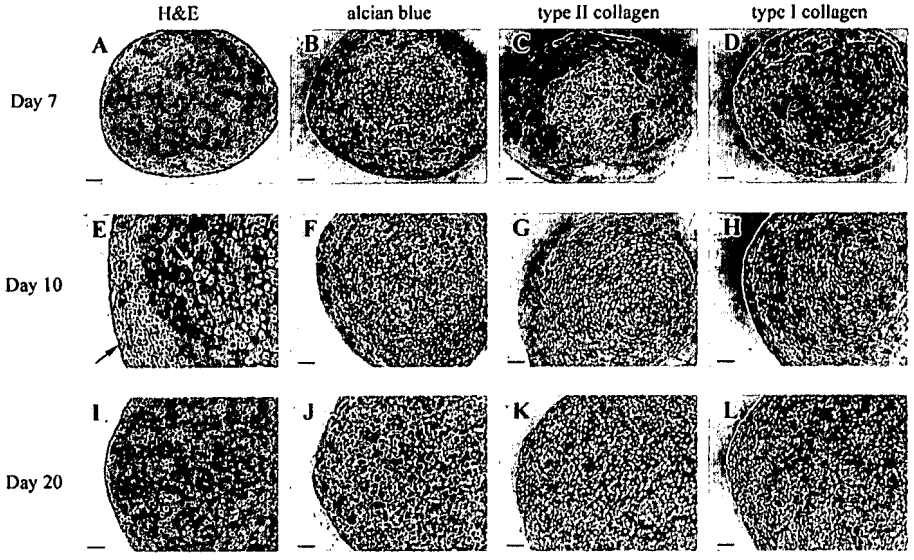

Figure 2. MSC cultured in pellet culture system on day 7 (**A–D**), on day 10 (**E–H**), and on day 20 (**I–L**) without treatment by TGF-β1. Hematoxylin and eosin staining (H&E) of the pellets (**A, E, I**); alcian blue staining (**B, F, J**); immunohistochemical staining for detection of type II collagen (**C, G, K**) and type I collagen (**D, H, L**). Positive metachromatic staining and immunoreactivity for type II collagen was detected on day 7 in the center of the pellet, which increased towards the surface of the pellet time depending. Stronger expression of type I collagen was detected on the surface and in the periphery layers of the pellet. On day 10, cells that showed appearance of hypertrophic chondrocytes were clearly identified in the center (white arrow), and elongated cells in the periphery layers (black arrow) of the pellets. Scale bar 100 μm.

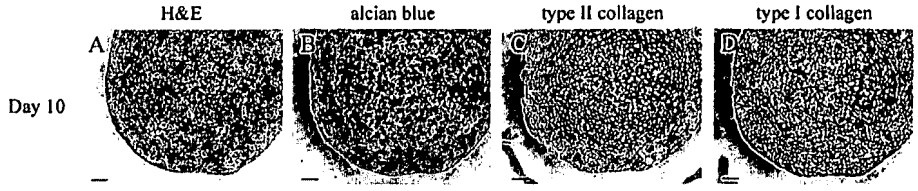

Figure 3. MSC cultured in pellet culture system on day 10 treated by 10 ng/mL TGF-β1. H&E staining of the pellets (**A**); alcian blue staining (**B**); immunohistochemical staining for detection of type II collagen (**C**) and type I collagen (**D**). There were no notable differences in histological and immunohistochemical examinations with pellets which have not been treated by TGF-β1. Scale bar 100 μm.

the cells in the superficial layer. Metachromatic staining and morphology of the cells suggested that a cartilaginous matrix had been synthesized. To confirm these findings, immunohistochemical analysis for type II collagen was carried out using mono-specific antibody. Positive immunostained areas corresponded to the metachromatic stained areas and comprised the entire pellets on day 20 (Fig. 2K). Type I collagen was detected in all of the pellet groups, and its intensity increased towards the surface of the pellets opposite of the expression of type II collagen (Fig. 2D, H, L). There were not any notable differences on immunohistochemical examination between the pellets treated by TGF-β1 in concentrations of 1 and 5 ng/mL (data not shown), pellets treated in concentrations of 10 ng/mL (Fig. 3), and pellets that were not treated (Fig. 2) during culturing of 20 days.

In situ hybridization

To verify results obtained from histological and immunohistochemical examination, in situ hybridization on the pellets cultured for 20 days was carried out. Type II collagen mRNA was strongly expressed in cells at the center of pellets and in the areas where pellets were densely organized (Fig. 4A). On the other hand, type I collagen mRNA was strongly expressed in the cells at the periphery of the pellets, and in less densely organized areas (Fig. 4B). Aggrecan mRNA was expressed throughout entire pellets with slightly more intensive expression at the centers (Fig. 4C). There were no notable differences in expression of mRNA between groups of pellets differing in concentrations of TGF-β1 (data not shown).

Measurement of mRNA expression

Total RNA was extracted from the MSC in pellet culture on day 1 and 20, and from MSC and chondrocytes cultured in monolayer manner for 20 days. There was no expression of chondrocyte-specific genes in pellets after one day of culturing or in the MSC from the monolayer culture (Fig. 5). Type II collagen and aggrecan mRNA expression were detected in pellets cultured for 20 days. In pellets that were not treated by TGF-β1, type II collagen mRNA was 1.7-fold higher compared to pellets cultured with TGF-β1 (Fig. 6). TGF-β1 did not have some influence on aggrecan mRNA expression in pellet-cultured MSC (Fig. 7). When type II collagen gene expression of MSC in pellet culture was compared to that of chondrocytes, the MSC exhibited 2.4-fold higher expression. Opposite of that, aggrecan mRNA expression in MSC from pellet culture was 3.1-fold lower than the expression level in the chondrocytes. Expression level of type I collagen mRNA of MSC in monolayer was 3.6-fold higher than that in pellet culture. TGF-β1 did not have some remarkable influence on expression of type I collagen in pellet culture. During culturing in monolayer manner, chondrocytes showed a normal shift from collagen type II to collagen type I gene expression (Fig. 5), but still kept the chondrogenic phenotype [27,28,32].

Discussion

In the present study, we demonstrated that expanded bovine MSC undergo chondrogenesis under appropriate culture conditions in a serum-free chemically defined medium. Independent, spontaneous chondrogenesis of bovine MSC in

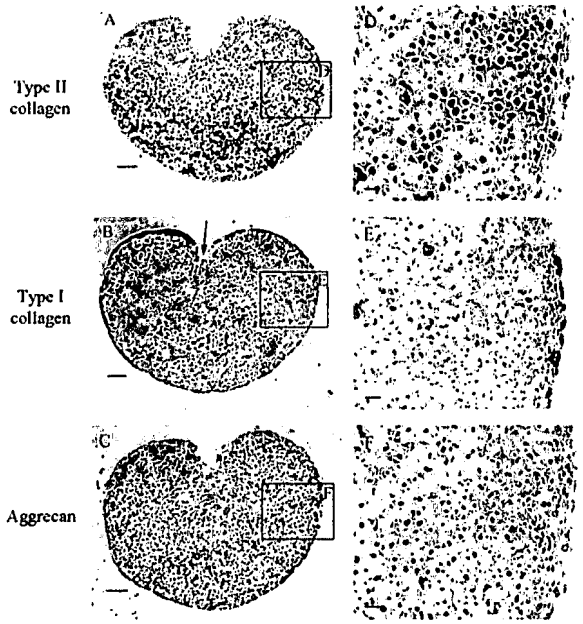

Figure 4. In situ hybridization of the pellet without treatment by TGF-β1 on day 20 for detection of type II collagen (**A, D**), type I collagen (**B, E**), and aggrecan mRNA(**C, F**). Type I collagen mRNA was strongly expressed in the cells on the surface of the pellet or in less densely organized areas (black arrow). Opposite of that, type II collagen and aggrecan mRNAs were expressed in the deeper part of the pellets. Scale bar (**A, B, C**) 50 μm; scale bar (**D, E, F**) 200 μm.

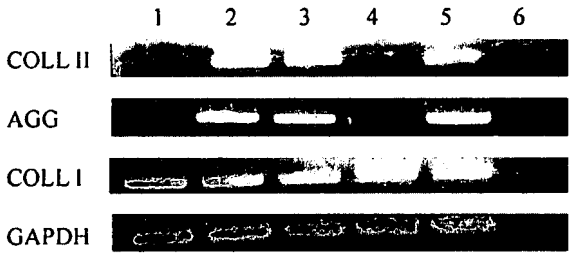

Figure 5. Electrophoresis of the PCR products from RT-PCR analysis of type II collagen (COLL II), aggrecan (AGG), type I collagen (COLL I), and GAPDH mRNA expression: (1) MSC in pellet culture on day 1; (2) MSC in pellet culture on day 20; (3) MSC in pellet culture on day 20 treated with 10 ng/mL TGF-β1; (4) MSC in monolayer on day 20; (5) chondrocytes in monolayer culture on day 20; (6) negative control (without reverse transcriptase). Type II collagen and aggrecan were not detected in the MSC cultured in monolayer for 20 days and in pellet culture for 1 day.

pellet culture occurred without addition of any external bioactive stimulators, namely TGF-β1, which was previously reported to be necessary [13–15,33]. The presence of metachromatic-staining matrix, the chondrocytic appearance of the cells, and detections of type II collagen and aggrecan mRNAs and proteins in the pellets showed that the tissue formed by these marrow-derived cells is cartilage.

Bovine MSC had spindle, bipolar to polygonal fibroblastic shape, and exhibited similar form to MSC isolated from other species [1,8–16]. On examination by phase contrast microscope, cell culture appeared to be homogeneous after the third or fourth changing of the medium, by the removal of nonadherent cells from hematopoietic lineage. Fluorescence-activated cell sorting (FACS) analysis of the cells from the first passage showed homogeneous cell size population, negative on CD4 (data not shown). Morphology and the fact that cells were able to undergo adipogenic and osteogenic differentiation in monolayer culture (data not shown), as well as chondrogenic differentiation in pellet culture, were the reasons for classifying them as MSCs [12,16].

Chondrogenesis was proven by the detection of sulfated GAG and strong immunoreactivity for type II collagen in

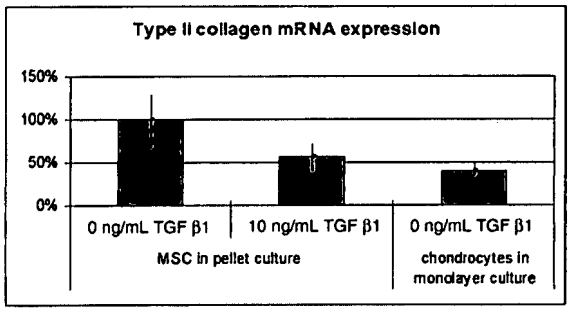

Figure 6. Quantitative analysis of type II collagen gene expression of MSC in pellet culture treated with 0 and 10 ng/mL TGF-β1, and in chondrocytes cultured in monolayer manner for 20 days. The expression level was normalized for the level of GAPDH mRNA. Expression of type II collagen in pellet culture without treatment by TGF-β1 was presented to be 100%. Data were evaluated from 4 independent experiments and shown mean ± SD.

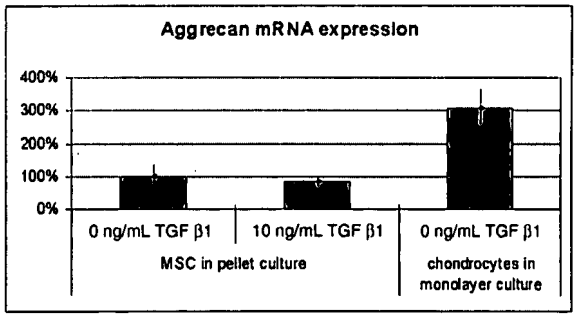

Figure 7. Quantitative analysis of aggrecan gene expression of MSC in pellet culture treated with 0 and 10 ng/mL TGF-β1, and in chondrocytes cultured in monolayer manner for 20 days. The expression level was normalized for the level of GAPDH mRNA. Expression of aggrecan in pellet culture without treatment by TGF-β1 was presented to be 100%.

the center of the pellets after 7 days of culturing. The positive stained area for chondrocytic markers increased with culturing time, and almost whole pellets were positively stained after 20 days culturing. MSC from most of the species undergo chondrogenesis under specific circumstances to satisfy the following conditions: high cell density, which allows cell-to-cell interaction analogous to those occurring in precartilage mesenchymal condensation during limb development; and the action of bioactive factors that stimulate chondrogenesis (e.g., TGF-β, BMP, FGF, IGF-I, and dexamethasone) [15]. The condensed condition can be obtained by pellet culture systems [33], which were sufficient external stimulants for bovine MSC to undergo chondrogenesis. In our study for preparations of pellets, we used 4 times larger numbers of cells [29] compared to previous reports [33–36]. The reason was to obtain pellet volume as large as possible, so that the cells present in the central region would be under higher pressure. Also, large pellets provide a larger number of cells to have better conditions for cell-to-cell interactions. However, we found that spontaneous chondrogenesis of bovine MSC did not depend on the initiating cell number used for pellet preparation (data not shown). Previously Reyes et al. also used 1×10^6 cells for pellet preparation, but they had to treat human MSC by TGF-β1 to induce chondrogenesis [29]. Present in situ hybridization clearly showed highest expression levels of type II collagen mRNA at the center region of the pellet where the cell density was higher, suggesting that condensed condition obtained by the pellet culture system is important for induction of chondrogenesis. Conversely, on the surface of the pellets, where the conditions look similar to monolayer culture, the cells expressed type I collagen and aggrecan, but no type II collagen (Fig. 4). In vitro monolayer cultivation of chondrocytes is accompanied by dedifferentiation with expression of collagen type I, which turns to redifferentiation of the same cells with an increase of collagen type II expression in three-dimensional cultures [37,38]. The importance of the culture condition in chondrogenesis, being independent of bioactive stimulators, has also been reported in rabbit periosteal explants cultured in agarose gels [39].

There are several possible explanations concerning the self-induced chondrogenesis in bovine MSC. One of them is the species-specific character of stem cells. MSC have been isolated and characterized from almost all of the experimental animals and humans, and exhibited some species-specific characteristics in each. For example, in ovine [40] and feline [14] MSC, ALP activity could not be detected during osteogenic differentiation; however, increase of ALP activity is a typical osteogenic phenotype for human and rat MSC [17,41]. In cultures of embryonic chick limb bud cells and rabbit periosteal explants, TGF-β1 induced the formation of cartilaginous tissue [42,43], whereas rat periosteal cell in pellet culture failed to undergo chondrogenesis by the treatment of TGF-β1 [44]. MSC derived from almost all species except rat [44] formed cartilage by the pellet culture in response to TGF-β1 and dexamethasone. In other animals, spontaneous chondrogenic differentiation of stem cells was reported to be only sporadic. Fortier et al. reported spontaneous chondrogenesis of equine MSC cultured in monolayer manner in culture medium supplemented with 10% FBS [12]. However, chondrogenesis did not occur in serum-free medium, which could not eliminate the influence of bioactive stimulants present in the FBS.

The mechanism of chondrogenesis without any bioactive stimulants observed in bovine MSC remains unknown and is the subject of our ongoing research. Most likely, the condensed culture condition is the first stimulant for inducing further autocrine/paracrine cytokine secretion on a cellular level, which proceeds to complete differentiation to the chondrocytes. However, it is not yet reliable to discuss which cytokine, or what other factors may play a role.

TGF-β1 treatment of bovine MSC in pellet culture decreased expression of type II collagen mRNA. The same effect of TGF-β1 has been reported on freshly isolated chondrocytes cultured in monolayer culture [16]. The effects of TGF-β1 on chondrocyte proliferation and synthesis of extracellular matrix proteoglycan and type II collagen is contextual and may be stimulatory or inhibitory, depending on the stage of cellular differentiation, growth condition, and the presence of other factors. TGF-β1 reduced matrix protein expression and inhibited DNA synthesis in freshly isolated chondrocytes, but stimulated collagen type II and aggrecan expression in long-term cultured cells [45]. In monolayer culture, chondrocytes changed their form from the polygonal one recognized immediately after isolation to the fibroblastic one observed after one week of culturing. During these transformations of cell shape, type II TGF-β receptors changed in size. Type II TGF-β receptors predominantly expressed in cultured chondrocytes are approximately 15 kDa smaller than those expressed in freshly isolated chondrocytes, and are postulated to be nonfunctional [45]. Long-term cultured chondrocytes have fibroblastic shapes similar to MSC expanded in monolayer for two passages, and both respond to stimulation with TGF-β1 by expression of type II collagen mRNA (our unpublished data). MSC in pellet culture changed their shape from fibroblastic to polygonal, in which case TGF-β1 actually had a suppressive effect on expression of type II collagen.

It has been previously reported that age of the donor had no relationship with the ability of the culture-expanded MSC to undergo chondrogenesis [46]. Some other stem cells with chondrogenic potential, derived from a different origin, such as human periosteum-derived cells from donors younger than 30 years, undergo focal chondrogenic differentiation in the first or second passage in monolayer culture [34,47,48]. De Bari looked for a possible explanation for losing potential for spontaneous chondrogenesis upon passaging in the cell selection, so that periosteum-derived cells responsible for the formation of chondrogenic nodules were overgrown by other cell subsets [34]. Physical and health conditions of the donors might have influence on the characteristics and potential of the stem cells. For example, human MSC isolated from patients with advanced osteoarthritis have reduced chondrogenic and adipogenic activities [46]. Considering these results, we should mention that bone marrow aspirates in our study were harvested from young animals (2 days to 6 months old), and could be one of the reasons for the observed high chondrogenic potential of the cells. In addition, we confirmed spontaneous chondrogenesis of bovine MSC isolated from adult donors (more than 4 years old), but comparing chondrogenic potentional of MSC harvested from young to that of adult animals was beyond the scope of our research.

In conclusion, we demonstrated that the species character must be considered in all experiments involving MSC. We have shown that the process of chondrogenic differentiation in bovine MSC appears to be different from other species, but further experimental work is necessary in order to be well understood. Finally, we made the first step in establishing a new animal experimental model for MSC research.

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